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Effect of ω-agatoxin-IVA on autonomic neurotransmission

Paul M. Lundy *, Robert Frew

Pharmacology and Therapeutics, Defence Research Establishment Suffield, Box 4000, Medicine Hat, Alberta, Canada T1A 8K6

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Abstract

ω-Agatoxin-IVA, a peptide from the venom of the funnel-web spider *Agelenopsis aperta* and a P type Ca^{2+} channel inhibitor, was examined for effects on responses to nerve stimulation in isolated autonomic neuroeffector preparations from the rabbit, guinea-pig and rat. Ca^{2+}-dependent, tetrodotoxin sensitive, noradrenergic excitatory responses of rabbit pulmonary artery, rat vas deferens, and anococcygeus muscles, and cholinergic guinea-pig myenteric plexus preparations (all highly sensitive to the N type Ca^{2+} channel inhibitor ω-conotoxin-GVIA) were unaffected by ω-agatoxin-IVA (100 nM). Similarly, the neurogenic response of rat bladder, which has cholinergic, and non-adrenergic non-cholinergic (NANC) excitatory components, and the NANC inhibitory response of rat jejunum (atropine 0.5 μM- and guanethidine 5.0 μM-treated), which are partially sensitive and insensitive to ω-conotoxin-GVIA, respectively, were unaffected by ω-agatoxin-IVA (100 nM). Neurogenic NANC inhibitory responses of the guinea-pig taenia caecum, and rat anococcygeus muscles (atropine- and guanethidine-treated, and tone raised with prostaglandin $F_{2\alpha}$), were also insensitive to ω-agatoxin-IVA. These results suggest that P type Ca^{2+} channels, if present, play an insignificant role in supplying the Ca^{2+} necessary for neurotransmitter release in the peripheral autonomic nervous system.

Key words: ω-Agatoxin-IVA; ω-Conotoxin-GVIA; Ca^{2+} channel (voltage sensitive); Autonomic nerve; Neurotransmission; Smooth muscle

1. Introduction

Voltage sensitive Ca^{2+} channels (VSCC's) provide a major route for elevation of the intracellular Ca^{2+} concentrations necessary to initiate neurotransmitter release. In the brain, several types of high threshold channels have been characterized by biophysical or pharmacological means. These channels have been designated by their sensitivity to inhibition by the dihydropyridines (L type), ω-conotoxin-GVIA (N type) and by either the polyamine funnel web spider toxin FTX (Llinas et al., 1989) or a peptide fraction from funnel web spider venom known as ω-agatoxin-IVA (P type) (Mintz et al., 1992a).

N and L type VSCCs have been identified in a number of peripheral nerves. It is generally accepted that N channels play a critical role in regulating the Ca^{2+} influx necessary for neurotransmitter release at peripheral nerve terminals (Hirning et al., 1988; Lundy and Frew, 1988; Maggi et al., 1988; Keith et al., 1989; DeLuca et al., 1990). A significant role for L channels has been much more difficult to demonstrate (Wessler et al., 1990), perhaps because these channels may be located at sites distant from the release sites, because of preferential distribution at nerve cell bodies (Miller 1987; Thayer et al., 1987), or because they activate preferentially according to stimulus type. As with central neurons and synaptosomes, evidence suggests that an additional novel Ca^{2+} channel may also be present in peripheral neurons. For example, various types of cultured neurons appear to contain an ω-conotoxin-GVIA, dihydropyridine resistant type of channel (Perney et al., 1986; Plummer et al., 1989; Regan et al., 1991; Scroggs and Fox, 1992; Xu and Adams, 1992; Uchitel et al., 1992). The biophysical and pharmacological properties of this channel suggest that it may be similar to the P type channel found in mammalian brain. This peripheral channel, however, has not been characterized, as one of the three types described above (L, N or P). In addition, certain peripheral

* Corresponding author. Tel. 403-544-4694, fax 403-544-3388.

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neurons are reported to be relatively insensitive to ω-conotoxin-GVIA (Maggi et al., 1988; De Luca et al., 1990), suggesting the possible presence of residual Ca^{2+} channels. Evidence for the existence of P type channels in autonomic preparations has been demonstrated in some tissues (Mintz et al., 1992a). However unlike the role P channels have on neurotransmitter release in mammalian brain (Defeo et al., 1992; Burke et al., 1993), no clear functional correlation has been identified in the periphery.

We report here the results of experiments performed to determine the existence and functional significance of P channels at various autonomic neuroeffector junctions.

2. Materials and methods

2.1. Tissue preparation

Sprague Dawley rats (150–200 g), Hartley guinea-pigs (300–350 g), and New Zealand White rabbits (1.5–2.0 kg) were killed. Smooth muscle preparations were carefully excised and placed in oxygenated Krebs-Henseleit solution of the following composition (mM) NaCl 116, KCl 5.4, $CaCl_2$ 1.5, $MgCl_2$ 1.2, NaH_2PO_4 1.2, $NaHCO_3$ 22 and D-glucose 11, at pH 7.4. Tissues were suspended in 5 ml isolated organ baths containing Krebs-Henseleit solution aerated with 95% O_2-5% CO_2 maintained at 37°C. One end of each tissue was fixed, and the other attached by thread to a Harvard smooth muscle transducer. Auxotonic recording of responses to electric field stimulation and to drugs were displayed on a Rikadenki chart recorder. All tissues were allowed to equilibrate for 60 min prior to drug testing.

2.2. Experimental protocols

Frequency response curves to electrical field stimulation (0.5–50 Hz, 1 ms, for 30 s every 300 s) were obtained using rabbit pulmonary artery ring preparations by means of parallel platinum electrodes connected to a Grass model S88 stimulator. Complete frequency-response curves, either in the presence or absence of ω-agatoxin-IVA or ω-conotoxin-GVIA, were performed on each preparation allowing 30 min between curves.

Guinea-pig ileum myenteric plexus preparations prepared as described by Paton and Zar (1968) were continuously stimulated using square wave pulses at 0.2 Hz (1.0 ms, supramaximal voltage). Excitatory responses of rat bladder strips (Carpenter, 1977), anococcygeus muscles (Gillespie and Tilmisany, 1976) and vas deferens (Swedin, 1971) were obtained using electrical field stimulation (trains of 1 ms pulses, 5 Hz for 1 s every 30 s, supramaximal voltage). Since both the prostatic (purinergic) and epididymal (noradrenergic) portions of the rat vas deferens are equi-sensitive to ω-conotoxin-GVIA (Maggi et al., 1988), no attempt was made to discriminate between these in our preparation of the vas deferens.

Non-adrenergic, non-cholinergic (NANC) inhibitory responses to electrical field stimulation (train 1 ms, 10 Hz, for 1 s every 100 s, supramaximal voltage) were obtained using guinea-pig taenia caecum (Burnstock et al., 1966), rat jejunum (Maggi et al., 1988), and rat anococcygeus muscles, pretreated with the muscarinic antagonist atropine (0.5 μM) and the adrenergic neuron blocker guanethidine (5 μM), and in which tone was induced by prostaglandin $F_{2\alpha}$ (0.5 μg/ml).

ω-Agatoxin-IVA (100 nM), or ω-conotoxin-GVIA (10–100 nM), were added to these electrically stimulated preparations following establishment of uniform responses to electrical field stimulation, and effects on the neurogenic response observed. The amplitude of the neurogenic response following drug addition was expressed as a percentage of the response amplitude immediately prior to drug addition.

2.3. Drugs

Drugs used in this study and their sources are as follows: ω-agatoxin-IVA (Research Biochemicals) or ω-conotoxin-GVIA (Peninsula Laboratories), were dissolved in distilled H_2O, and aliquots stored at −20°C until use. Prostaglandin $F_{2\alpha}$ tromethamine salt (Upjohn), (−)-noradrenaline bitartrate, atropine sulfate, guanethidine sulfate and TTX were purchased from Sigma (St. Louis).

2.4. Statistics

Data are expressed as means ± S.E.M. Significance of differences were assumed if $P < 0.05$ in a non-paired Student's t-test.

3. Results

Frequency-response curves to stimulation of sympathetic perivascular nerves in rabbit pulmonary artery ring preparations were not affected by ω-agatoxin-IVA (100 nM), but were shifted to the right by the N channel blocker ω-conotoxin-GVIA (30 nM) (Fig. 1).

Neurogenic cholinergic excitatory responses of the guinea-pig myenteric plexus, and noradrenergic excitatory responses of the anococcygeus muscle, were unaffected following a 30 min exposure to, and in the continued presence of ω-agatoxin-IVA (100 nM) (Table 1). In the untreated rat vas deferens, repeated stimulation results in a gradual attenuation of the

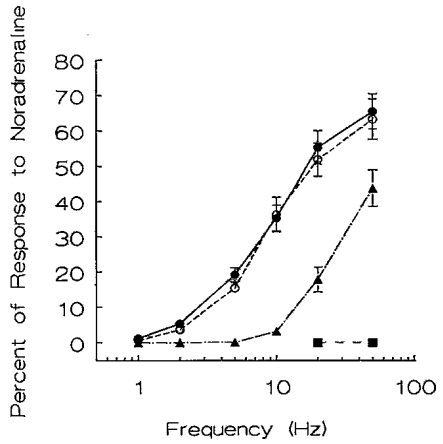

Fig. 1. Effect of ω-agatoxin-IVA and ω-conotoxin-GVIA on responses to electric field stimulation in rabbit pulmonary artery rings. In the absence of (●), or following a 30 min pretreatment and in the continuing presence of ω-agatoxin-IVA 100 nM (○), ω-conotoxin-GVIA 30 nM (▲) or tetrodotoxin 1 μM (■). Each point represents mean ± S.E.M. ($n = 6$), expressed as a percent of the response to 1 μM noradrenaline.

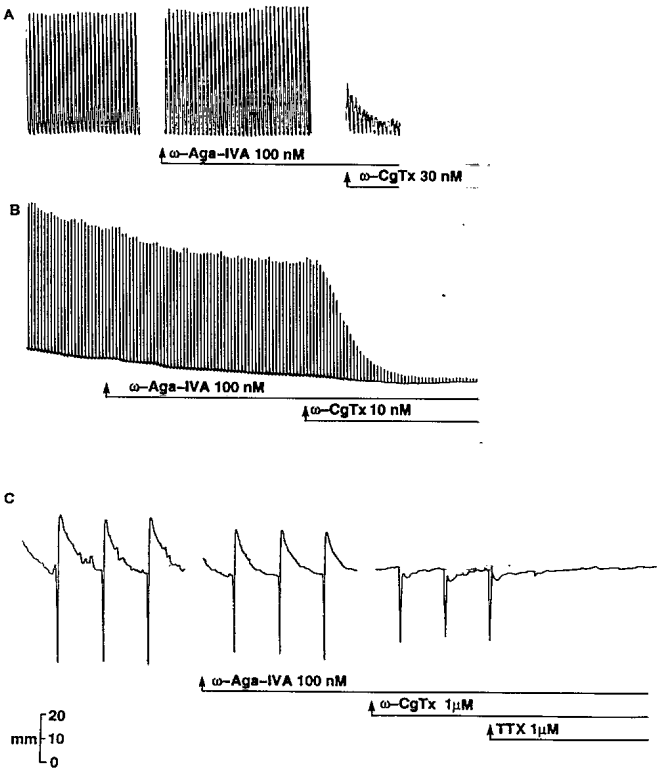

Fig. 2. Effect of ω-agatoxin-IVA and ω-conotoxin-GVIA on responses to electric field stimulation. (A) Cholinergic 'twitch' responses of the guinea-pig myenteric plexus preparation to continuous stimulation at 0.2 Hz. Untreated (left); following a 30 min pretreatment, and in the continued presence of ω-agatoxin-IVA (ω-Aga-IVA) 100 nM (middle), and in the additional presence for 30 min of ω-conotoxin-GVIA (ω-CgTx) (right). (B) Noradrenergic rat vas deferens (train 1 ms pulses, 5-Hz for 1 s every 30 s). ω-Agatoxin-IVA was added for 30 min, followed by ω-conotoxin-GVIA. (C) NANC inhibitory responses to nerve stimulation (train of 1 ms pulses, 10 Hz for 1 s every 100 s) in guinea-pig taenia caecum in the absence (left), and presence (middle) of ω-agatoxin-IVA, and additional presence of ω-conotoxin-GVIA and tetrodotoxin (TTX) (right). Tone was raised using prostaglandin $F_{2\alpha}$ (0.5 μg/ml) in guanethidine (5 μM)- and atropine (0.5 μM)-treated taenia caecum.

neurogenic response due to an endogenous prostaglandin-induced inhibition of transmitter release from sympathetic nerves (Swedin, 1971). A slight, but statistically insignificant inhibition of the neurogenic response following ω-agatoxin-IVA treatment in the vas deferens (Table 1, Fig. 2b), rather than indicating inhibition by ω-agatoxin-IVA, appears to be due to this phenomenon. Neurogenic excitatory responses in all these tissues were sensitive to low concentrations of ω-conotoxin-GVIA studied sequentially on the same tissues (Table 1).

NANC inhibitory responses to electrical field stimulation, in atropine- and guanethidine-treated rat anococcygeus muscles, and guinea-pig taenia caecum, were not significantly affected by ω-agatoxin-IVA (100 nM) (Table 1). Typical traces showing the lack of effect of ω-agatoxin-IVA on cholinergic, noradrenergic and NANC responses are shown in Fig. 2a,b,c, respectively.

Sensitivity of NANC nerves to ω-conotoxin-GVIA varies widely between species and tissues. The rat jejunum, in particular, has been reported to be totally insensitive to ω-conotoxin-GVIA, while the rat urinary bladder is only partially sensitive (Maggi et al., 1988;

Table 1
Effects of ω-agatoxin-IVA and ω-conotoxin-GVIA on responses to electric field stimulation in autonomic neuroeffector preparations

Treatment	Guinea pig		Rat				
	Myeneteric plexus [a]	Taenia caecum [c]	Vas deferens [b]	Bladder [d]	Anococcygeus [b]	Anococcygeus [c]	Jejunum [c]
Untreated	100	100	100	100	100	100	100
ω-Agatoxin-IVA							
100 nM	96.7 ± 3.2	103.3 ± 1.2	84.5 ± 6.7	92.3 ± 2.0	89.4 ± 5.4	109.9 ± 14.5	102.3 ± 4.6
ω-Conotoxin-GVIA							
10 nM	8.4 ± 1.2 [e]	–	3.1 ± 0.8 [e]	–	2.1 ± 2.1 [e]	–	–
50 nM	–	79.9 ± 3.9	–	–	–	36.5 ± 8.3	–
100 nM	–	–	–	42.4 ± 2.1	–	–	66.7 ± 5.2

[a] Cholinergic; [b] noradrenergic; [c] NANC inhibitory; [d] NANC excitatory. Values represent means ± S.E.M. obtained in the absence, or following a 30 min pretreatment, and in the continued presence of the peptide in tissues from 3–5 animals. Stimulation parameters are as described in Methods. [e] Significantly different from ω-conotoxin-GVIA treated NANC values ($P < 0.05$). ω-Agatoxin-IVA values not significantly different from untreated values ($P > 0.05$). Values shown represent percent of control response.

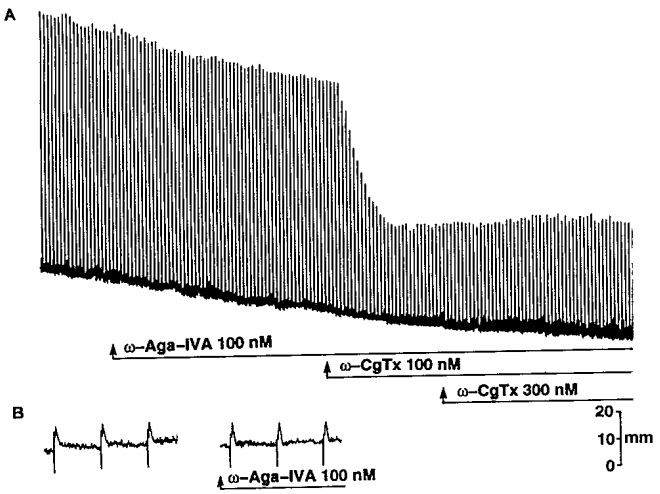

Fig. 3. Effect of ω-agatoxin-IVA on neurogenic responses of (A) rat bladder strip (train of 1 ms pulses, 5 Hz for 1 s every 30 s) and (B) rat jejunum (train of 1 ms pulses, 5 Hz for 1 s every 100 s). The jejunum was treated with guanethidine (5 μM) and atropine (0.5 μM).

De Luca et al., 1990). This relative insensitivity was confirmed in the present study where a residual component of the response to electrical field stimulation was evident ($42.4 \pm 2.1\%$ and $66.7 \pm 5.2\%$ of control responses) in the rat bladder, and jejunum, respectively in the presence of 100 nM ω-conotoxin-GVIA. ω-Agatoxin-IVA (100 nM) had no effect on NANC responses to electrical field stimulation (Table 1). Typical traces showing effects of ω-agatoxin-IVA on responses to electrical field stimulation in rat bladder and jejunum are shown in Fig. 3a,b. Furthermore, NANC inhibitory responses to electrical field stimulation in guinea-pig taenia caecum were also found to be particularly insensitive to ω-conotoxin-GVIA and to ω-agatoxin-IVA (Table 1, Fig. 2c). However, the rebound contraction following cessation of electrical field stimulation was abolished by ω-conotoxin-GVIA (Fig. 2c). This apparent ω-conotoxin-GVIA insensitivity of NANC responses in guinea-pig taenia caecum which we observed is in agreement with the finding of De Luca et al. (1990) which showed NANC responses to be less sensitive than cholinergic responses to ω-conotoxin-GVIA in this tissue. Thus a relative insensitivity to ω-conotoxin-GVIA was evident in NANC-mediated responses to electrical field stimulation in this study, as compared to noradrenergic and cholinergic responses which are highly sensitive to ω-conotoxin-GVIA (see Table 1). All responses to electrical field stimulation, excitatory or inhibitory, in all tissue types, were inhibited by tetrodotoxin (1 μM) confirming that responses were of neuronal origin. The effect of ω-agatoxin-IVA at a higher concentration of 1 μM was tested on the response to electrical field stimulation of one preparation of each tissue type, inhibition was $< 10\%$ in all cases (not shown).

4. Discussion

The present study offers no evidence of a significant role for P type VSCC's in peripheral autonomic neurotransmission. In contrast to the N type channel inhibitor ω-conotoxin-GVIA, the specific P channel inhibitor ω-agatoxin-IVA, at concentrations which fully inhibit Ca^{2+} influx in rat brain (Mintz et al., 1992b), had no effect on autonomic neurotransmission. The effectiveness of ω-conotoxin-GVIA in blocking noradrenergic and cholinergic neurogenic responses is well documented and suggests a major role for N channels in supplying Ca^{2+} necessary for neurotransmitter release (see Introduction). The existence of ω-conotoxin-GVIA, dihydropyridine insensitive VSCC's has been reported in some cultured autonomic preparations (Plummer et al., 1989; Boland and Dingledine, 1990; Regan et al., 1991; Scroggs and Fox, 1992; Xu and Adams, 1992) but not in other sympathetic (Mintz et al., 1992b; Mintz and Bean, 1993), or parasympathetic preparations (Franklin and Willard, 1993).

Peripheral dihydropyridine, ω-conotoxin-GVIA resistant Ca^{2+} channels remain to be fully characterized. It is reasonable, based on their biophysical characteristics to suggest that they may be P type channels. However, the negligible effect of ω-agatoxin-IVA on the noradrenergic excitatory responses of electrically stimulated rabbit pulmonary arteries, rat vas deferens, and rat anococcygeus muscles suggests an absence of functional P channels on sympathetic nerve terminals in these vascular and non-vascular peripheral tissues. This view is supported by previous biophysical and pharmacological data suggesting that N channels are exclusively responsible for the Ca^{2+} influx responsible for noradrenaline release from sympathetic nerves (Hirning et al., 1988; Plummer et al., 1989; De Luca et al., 1990; Lundy and Frew, 1993). It is also in accord with other results (Mintz et al., 1992a), which failed to demonstrate any effect of ω-agatoxin-IVA on Ca^{2+} currents in cultured sympathetic neurons.

The neurogenic cholinergic twitch response of the guinea-pig ileum was also unaffected by ω-agatoxin-IVA. Functional ω-conotoxin-GVIA sensitive, N type channels have been previously identified in this tissue (Lundy and Frew, 1988, 1993; De Luca et al., 1990), and ω-conotoxin-GVIA has been shown to inhibit Ca^{2+} currents in cultured parasympathetic neuronal preparations in vitro (Xu and Adams, 1992; Aibara et al., 1992; Franklin and Willard, 1993). This observed inability of ω-agatoxin-IVA to inhibit cholinergic responses in guinea-pig ileum is supported by a previous study in which neurogenic responses in this tissue were insensitive to the P channel inhibitor synthetic funnel-web spider toxin (FTX) (Lundy and Frew, 1993). It is also in accord with results which suggested the absence of dihydropyridine, ω-conotoxin-GVIA insensitive high

threshold channels in cultured myenteric neurons (Franklin and Willard, 1993).

In a manner similar to noradrenergic and cholinergic transmission, NANC inhibitory transmission is presumed to be dependent on the influx of extracellular Ca^{2+} (Gillespie and Tilmisany 1976; Garthwaite 1991), which is reduced by ω-conotoxin-GVIA (De Luca et al., 1990; Rand, 1992). The ineffectiveness of ω-agatoxin-IVA in inhibiting NANC inhibitory responses to nerve stimulation in the rat anococcygeus muscle, rat jejunum and guinea-pig taenia caecum suggests that P channels also have no demonstrable role in supplying the Ca^{2+} necessary for release of the inhibitory NANC transmitter. It is not clear in NANC transmission, particularly in those tissues where nitric oxide is the transmitter (for review see Rand 1992) that exocytotic release occurs in a manner similar to that of the conventional neurotransmitters, or whether extracellular Ca^{2+} is required for nitric oxide release as well as nitric oxide synthesis (Garthwaite, 1991).

ω-Agatoxin-IVA inhibits Ca^{2+}-dependent, ω-conotoxin-GVIA insensitive neurotransmitter release in rat brain, defining a role for P type Ca^{2+} channels in neurotransmission in the central nervous system (Defeo et al., 1992; Burke et al., 1993). Similarly, ω-conotoxin-GVIA resistance of certain NANC nerves suggests a role for P type Ca^{2+} channels. The ineffectiveness of ω-agatoxin-IVA in inhibiting neurotransmitter release in these preparations in particular, suggests the possible existence of novel Ca^{2+} channels which are neither L, N nor P type. It cannot be precluded, however, that Ca^{2+} influx may not be required for ω-conotoxin-GVIA-insensitive neurotransmitter release.

In marked contrast to the established role for P type Ca^{2+} channels in the central nervous system, they do not appear important in supplying the Ca^{2+} necessary for neurotransmitter release in noradrenergic, cholinergic or NANC transmission in peripheral autonomic nerves. The lack of demonstrable effects of ω-agatoxin-IVA on neurotransmission does not preclude their presence either at, or spatially distinct from the nerve terminals, but suggests only a marginal role in neurotransmitter release, which contrasts with the significant role of ω-agatoxin-IVA sensitive P channels on neurotransmitter release in the central nervous system (Defeo et al., 1992; Burke et al., 1993). Our results add support to existing data indicating N channels as the major route by which Ca^{2+} is translocated into the nerve terminal to initiate transmitter release in the autonomic nervous system.

The fact that in the mammalian central nervous system, P type channels play a significant role relative to N channels, while displaying an insignificant role peripherally, raises the possibility of differential drug targeting of central and peripheral Ca^{2+} channels in the treatment of various pathological conditions such as epilepsy and stroke on one hand, and hypertension on the other.

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