British Journal of Pharmacology (1996) 118, 797-803

Inhibition of neuromuscular transmission in the myenteric plexus of guinea-pig ileum by ω-conotoxins GVIA, MVIIA, MVIIC and SVIB

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1 The effects of a number of Ca^{2+} channel blockers on the transmural electrical stimulation or receptor agonist-elicited contractile responses of guinea-pig ileum were compared.

2 ω-Conotoxins (MVIIA, GVIA, SVIB and MVIIC), but not ω-agatoxin IVA, completely blocked the twitch responses evoked by low frequency (0.1 Hz) transmural stimulation without inhibition of the contractures evoked by exogenous acetylcholine. The concentration-inhibition curves were shifted by changes of external Ca^{2+}.

3 The tetanic contractures produced by a high frequency (30 Hz) train of stimulation were inhibited by ω-conotoxins by only 25-30%, except for ω-conotoxin MVIIC, which produced about 55% inhibition, all significantly less than that produced by atropine (about 70%) or tetrodotoxin (about 85%). Combinations of ω-conotoxins did not produce additive inhibitory effects.

4 The four ω-conotoxins as well as atropine produced similar partial inhibition (53-62%) of the contractures evoked by dimethylphenylpiperazinium, while tetrodotoxin inhibited the contracture completely.

5 Nifedipine and Ni^{2+} depressed the nerve stimulation-evoked twitch response and tetanic contracture as well as acetylcholine contracture.

6 These observations suggest that, in the myenteric plexus, a subset of N-type Ca^{2+} channel dominates under low frequency stimulation, while high frequency stimulation may recruit additional channels and non-cholinergic pathways.

Keywords: ω-Agatoxin; ω-conotoxin; acetylcholine release; Ca^{2+} channel; myenteric plexus; parasympathetic nerve

Introduction

Calcium ions play an essential role in the process of excitation-secretion coupling (Katz & Miledi, 1965; Dodge & Rahamimoff, 1967). Under physiological conditions, the nerve action potential elicits a transient, spike-like depolarization of nerve terminals which, in turn, opens voltage-gated Ca^{2+} channels enabling calcium ions to enter and initiate neurotransmitter release. Studies from molecular neurobiology have revealed a diversity of Ca^{2+} channel subtypes (Hofmann *et al.*, 1994; Varadi *et al.*, 1995). The voltage-gated Ca^{2+} channels are subdivided into T-, L-, N-, O-, P-, Q- and R-types based on their electrophysiological and pharmacological characteristics (Zhang *et al.*, 1993; Randall & Tsien 1995). The N-, P- and Q-type Ca^{2+} channels (and L-channel in particular cases, Gandía *et al.*, 1995) have been identified as being closely associated with transmitter release, whilst the role of the other channel subtypes in transmitter release is less clear (Llinás *et al.*, 1992; Olivera *et al.*, 1994). Many lines of evidence suggest that the voltage-gated Ca^{2+} channels have different presynaptic distributions and multiple Ca^{2+} channel subtypes may coexist to support neurotransmitter release (Dunlap *et al.*, 1995; Elliot *et al.*, 1995; Miljanich & Ramachandran, 1995).

The identification of a particular functional Ca^{2+} channel subtypes in intact tissues is greatly assisted by the use of polypeptide toxins, which, by binding to specific channel molecules, interfere with the release of neurotransmitters and the effector response. In the mammalian peripheral nervous system, the Ca^{2+} channel subtypes responsible for neurotransmitter release appear to differ from tissue to tissue. For instance the N-channel is mainly involved in the release of noradrenaline from sympathetic fibres (Hirning *et al.*, 1988) and the release of acetylcholine from cholinergic nerves in the gastrointestinal tract (de Luca *et al.*, 1990; Boot, 1994), while for the release of acetylcholine from motor neurones innervating skeletal muscle the P-type seems most important (Bowersox *et al.*, 1995; Hong & Chang, 1995b). As for non-adrenergic, non-cholinergic neurotransmission, the participation of both N- and Q-type Ca^{2+} channels has been suggested (Frew & Lundy, 1995).

The aim of this study was to explore how ω-conotoxins targeting subsets of N- (with ω-conotoxins GVIA, MVIIA, MVIIC and SVIB) and OPQ-channels (with ω-agatoxin IVA and ω-conotoxin MVIIC) affect the myenteric plexus neuroeffector transmission of guinea-pig ileum activated by electrical stimulation or a nicotinic ganglionic stimulant in order to shed more light on the synaptic transmission and the Ca^{2+} channel subtypes involved.

Methods

Ileum preparation

Segments about 1 cm long of the distal portion of ileum were isolated from guinea-pigs (150-250 g, Hartley strain). An axial platinum rod was inserted into the lumen and a four-turn platinum wire was coiled around the ileum serve as stimulating electrodes. The mounted preparations were suspended in Tyrode solution (composition in mM: NaCl 137, KCl 2.8, $MgCl_2$ 1.1, $CaCl_2$ 1.8, NaH_2PO_4 0.33, $NaHCO_3$ 11 and dextrose 11.2), maintained at 37°C and continuously gassed with 95% O_2 and 5% CO_2 unless otherwise indicated. Ilea were equilibrated for 40-60 min under a resting tension of 0.5 g.

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The activity of the longitudinal muscle was measured isometrically with a strain gauge transducer (HSE, Germany). The signals were digitized with a waveform recorder (Gould, U.K.) for analyses of the magnitude and the area of contractions.

Electrical and chemical stimulations

Electrical transmural stimulation of the myenteric plexus was performed with rectangular pulses (0.6 ms duration and supramaximal voltage) at 0.1 Hz for single stimulations, or at 3, 10 or 30 Hz for 5 s every 15–20 min for trains of high frequency stimulation. Dimethylphenylpiperazinium (DMPP) and acetylcholine were applied to stimulate, respectively, the nicotinic receptors of intramural nerves and the postsynaptic muscarinic receptors. During the equilibration period, two to three consecutive contracture responses were elicited by acetylcholine, DMPP or trains of high frequency stimulation as controls. Usually, the profile of the respective contracture could be reproduced quantitatively provided that an interval of 15–20 min was allowed between the specific manoeuvres. When the two contractures evoked by the same manipulation varied more than 15%, the preparations were discarded.

Statistics

The inhibitory effects of toxins on the evoked contractile forces were calculated as a percentage of the respective control. The IC_{50} values were estimated by linear regression from the log concentration-inhibition curves. Results were collected from 3–6 guinea-pigs, each providing 2–3 segments, and expressed as means ± s.e. mean. Differences between means were assessed by Student's *t*-test and *P* values <0.05 were considered statistically different.

Chemicals

The peptide toxins (ω-agatoxin IVA, ω-conotoxins GVIA, MVIIA, MVIIC and SVIB) were purchased from the peptide Institute (Japan).

Results

Effects of field stimulation on guinea-pig ileum

The ileum preparation generated stable twitch responses on transmural field stimulation at 0.1 Hz. The twitch amplitude decreased by 38±2% in low Ca^{2+} (0.9 mM) Tyrode, while it increased by 42±4% in high Ca^{2+} (7.2 mM) Tyrode. Twitches were abolished reversibly by a low concentration of tetrodotoxin (0.3 μM) or atropine (0.1 μM), indicating that the effects of field stimulation were mediated mostly by excitation of intramural nerves and release of endogenous acetylcholine. At stimulation frequency higher than 0.3 Hz, twitch responses decreased progressively and it was difficult to reach consistent steady responses. Hence, in the following experiments, twitch responses were evoked at 0.1 Hz. Compared with the single twitch response, a train of high frequency stimulation (3–30 Hz for 5 s) evoked an enhanced tetanic contracture: the magnitude increased to 131±7% (3 Hz) to 245±29% (30 Hz) and the area of contracture to 158±9% (3 Hz) to 922±48% (30 Hz, $n=7$). The tetanic contractures evoked by low frequency stimulation (3 Hz) were abolished by tetrodotoxin (0.3 or 1 μM) and atropine (1 μM), but those evoked by high frequency stimulation (30 Hz) were inhibited incompletely and atropine produced a lesser degree of inhibition than did tetrodotoxin (Table 1).

Effects of toxins on contractions induced by transmural stimulation

The twitch responses evoked by single stimulations were inhibited by the conus polypeptides ω-conotoxins GVIA, MVIIA, MVIIC & SVIB (Figure 1). A comparison of the inhibitory effects is shown in Figure 2 and the estimated IC_{50} and IC_{90} are listed in Table 2. It is evident that, although there were considerable differences in the inhibition potency; all the ω-conotoxins inhibited the twitch responses completely with parallel concentration-inhibition curves. Interestingly, as the evoked twitches became progressively depressed by the conopeptides, the ileum preparation developed spontaneous contractions (Figure 1). The inhibition induced by ω-conotoxins MVIIA and GVIA was slightly reversible after washing. In contrast, the inhibitory action of ω-conotoxin MVIIC waned even without washing and twitches recovered more than 80% after wash out of the toxin. The inhibitory activities of the ω-conotoxins were more potent in low Ca^{2+} media but were less potent in high Ca^{2+} Tyrode solution as exemplified in Figure 3 for ω-conotoxins MVIIA and MVIIC. In a few preparations (7 out of 58), high concentrations of ω-conotoxins MVIIA and GVIA (100 nM) or SVIB and MVIIC (1000 nM) produced an initial transient elevation of basal tone and enhancement of twitch amplitude before the subsequent rapid blockade of twitches (Figure 4). The inhibitory activities of the ω-conotoxins were not reduced in preparations treated with 8-phenyltheophylline, phentolamine and naloxone (up to 3 μM, not shown). The spider toxin, ω-agatoxin IVA, produced weak

Table 1 Inhibitions of high frequency stimulation- or DMPP-induced contractures

		Inhibition of contracture area (%)[a]				
		Train of stimulation (Hz)			*DMPP* (μM)	
Treatment	*μM*	*3*	*10*	*30*	*10*	*30*
Tetrodotoxin	1	100	93±3*	83±3*	100*	97±2*
Atropine	1	100	86±3*	69±4*	73±3	62±4
ω-Conotoxin						
MVIIA	3	97±2	64±4	31±3	72±3	57±4
GVIA	3	100	59±4	26±3	69±3	60±4
MVIIC	3	100	83±3**	54±4**	68±3	63±3
SVIB	3	93±2	67±3	29±3	65±3	53±3
MVIIC + GVIA		100	85±2**	51±3**	70±3	61±4
MVIIA + SVIB		100	64±3	28±3	69±3	54±4

[a]Guinea-pig ileum preparations were stimulated either with trains of electrical pulse at 3, 10 or 30 Hz for 5 s or with a ganglionic stimulant DMPP at 10 or 30 μM for 30 s. Data were pooled from 9–17 segments (n = 3–6).
*$P<0.05$ compared with the inhibitions produced by the four ω-conotoxins.
**$P<0.05$ compared with the inhibitions produced by ω-conotoxins GVIA, MVIIA and SVIB.
Note that all blockers produced a lesser degree of inhibition at a higher frequency of stimulation.

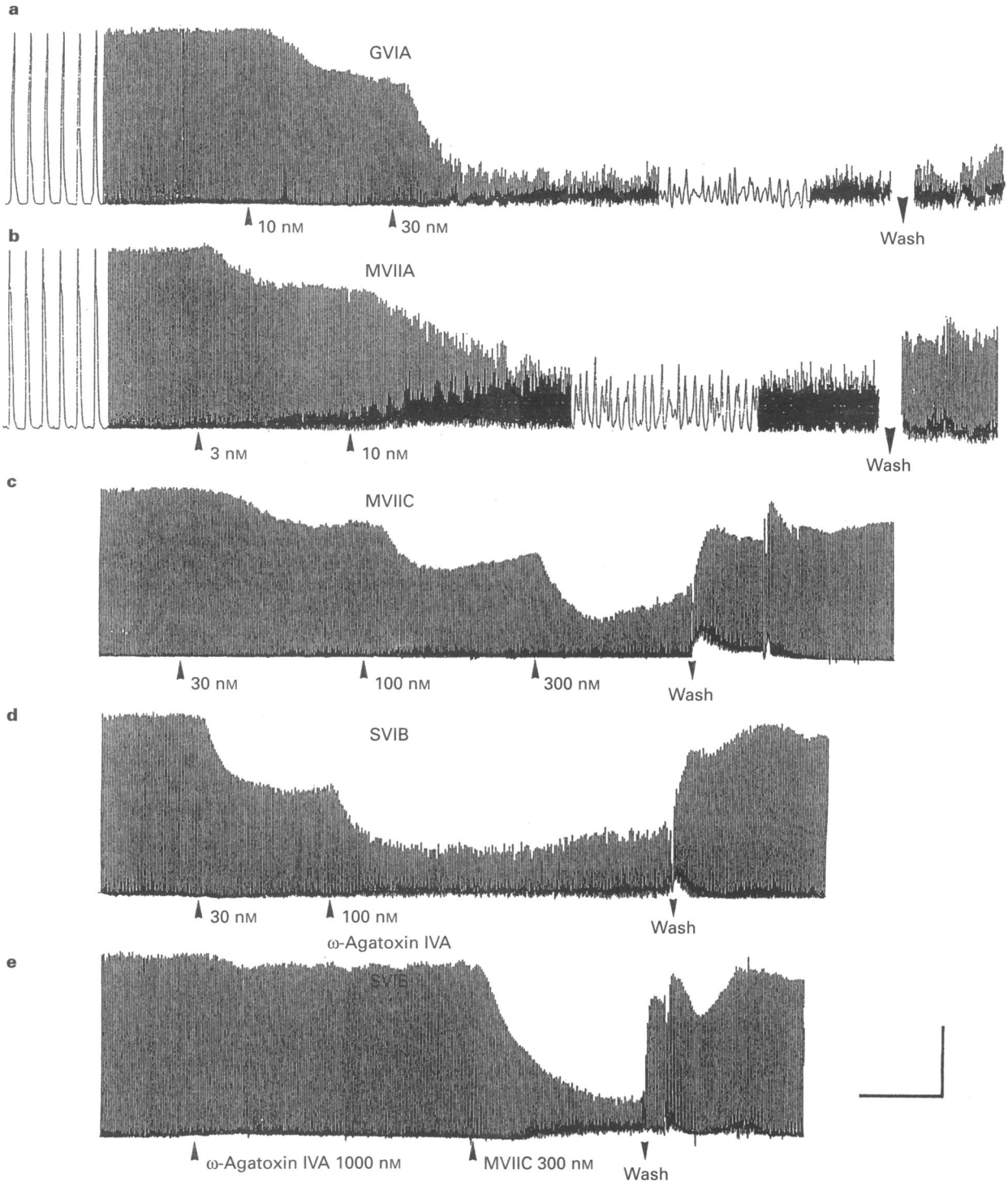

Figure 1 Inhibition of twitch response by ω-conotoxins: guinea-pig ilea were transmurally-stimulated at 0.1 Hz and treated, respectively, with ω-conotoxin GVIA (a), MVIIA (b), MVIIC (c), SVIB (d), and ω-agatoxin IVA + ω-conotoxin MVIIC (e). Preparations developed prominent spontaneous activities as twitch responses were depressed (a and b). Also note the different degrees of recovery after washout of toxins (downward arrow-heads, the large downward arrow-heads in (a) and (b) represent a time interval of 30 min). Calibrations: 1 g and 500 s or 50 s (for the expanded time scale in a and b).

inhibitions (16±3%) only at high concentrations (300 or 1000 nM). These concentrations, which are a hundred fold higher than those required to block the P-type channel, did not affect the inhibitory activities of the ω-conotoxins (Figure 1e).

In comparison to the inhibition of the twitch responses, the tetanic contractures induced by high frequency stimulation were inhibited to a lesser extent by the ω-conotoxins (Figure 5) and the higher the stimulation frequency the less was the inhibition produced (Table 1). At 30 Hz stimulation, atropine inhibited the contracture by about 70% whereas ω-conotoxins GVIA, MVIIA and SVIB produced only 25–30% inhibition and ω-conotoxin MVIIC, 54%. The maximal degree of inhibition produced by combinations of any two conotoxins was not additive.

Effects of Ni^{2+} and nifedipine

Ni^{2+} (0.1–5 mM) induced a short term (1–5 min) elevation of muscle tone (0.1–1.1 g, $n=4$). At low concentrations (0.1 or 0.3 mM), Ni^{2+} produced a transient (1–3 min) inhibition of

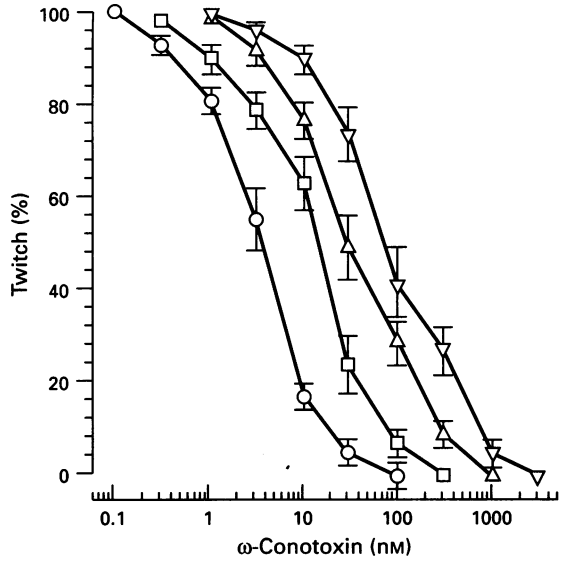

Figure 2 Concentration-dependent inhibition of twitch response by ω-conotoxins: ileum preparations were transmurally-stimulated at 0.1 Hz. ω-Conotoxins were applied cumulatively (○: MVIIA; □: GVIA; △: SVIB; ▽: MVIIC). The effects of toxins at each concentration were observed for 20 min from which the maximal inhibitions were plotted. $n=3-7$.

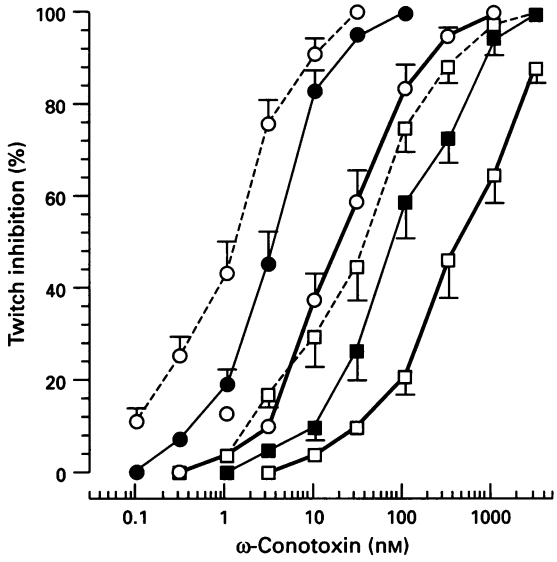

Figure 3 Effects of calcium on the concentration-inhibition curve: ileum preparations were stimulated at 0.1 Hz in low (0.9 mM, dotted lines), normal (1.8 mM, solid symbols) or high (5.4 mM, thick line) Ca^{2+} Tyrode solution. ω-Conotoxins were applied cumulatively (○, ●: MVIIA; □, ■: MVIIC. $n=4-5$.

Table 2 Relative potencies of ω-conotoxins against the single and train of stimuli-induced contraction of guinea-pig ileum

ω-Conotoxins	Twitch response[a] IC_{50} (nM)	IC_{90} (nM)	Tetanic contracture[b] IC_{50} (nM)	Affinity (IC_{50})[c]
MVIIA	3.5±0.6	18±2	>>3000	10 pM~1 nM
GVIA	16±2	93±6	>>3000	100 pM
SVIB	37±4	311±19	>>3000	4 nM
MVIIC	95±8	856±43	~3000	10 pM~1 nM

[a]Evoked at 0.1 Hz; [b]Evoked with a train of pulse (30 Hz for 5 s); [c]The high affinity binding to central neurones (cited from Hillyard *et al.*, (1992); Ramilo *et al.* (1992); Adams *et al.* (1993); Kristipati *et al.* (1994)).

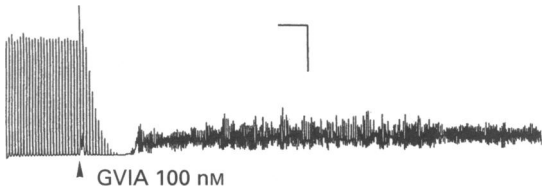

Figure 4 The atypical effect of ω-conotoxin on twitch response: the ileum preparation was transmurally-stimulated at 0.1 Hz throughout. Note the transient 'facilitatory' effect induced by a high concentration of ω-conotoxin GVIA (100 nM). Calibrations: 1 g and 100 s.

twitch responses by 29±4%. Twitches then recovered gradually to the control level as the basal tone returned. At higher concentrations, Ni^{2+} produced concentration-dependent reversible inhibitions (IC_{50}: 0.79±0.15 mM, Figure 6). The dihydropyridine Ca^{2+} channel blocker, nifedipine, inhibited twitches with an IC_{50} values of 0.044±0.009 μM ($n=4$), about 4 orders more potent than Ni^{2+}. However, part of the twitch responses (19±3%, $n=6$) remained uninhibited even when treated with a high concentration of nifedipine (1 μM). Ni^{2+} and nifedipine reduced twitches and tetanic contractures in a parallel fashion (Figure 6), in contrast to the disproportionate inhibitions produced by ω-conotoxins.

Effects of toxins on DMPP-contracture

Instead of applying electrical stimulation, the neurotransmitter release from myenteric plexus was triggered by DMPP (10 or 30 μM, 30 s). On application of the ganglionic stimulant, the ileum exhibited a two-phase contraction: an initial phasic contracture lasting for about 10 s, the amplitude of which was 50–120% that of the twitch response induced by field stimulation. Thereafter the contracture declined to a plateau level which was 27±6% that of the initial phase (Figure 7). Tubocurarine (3 μM) and tetrodotoxin (1 μM) completely blocked the DMPP-contracture, in agreement with the proposition that the effects of the ganglionic stimulant were mediated by excitation of nicotinic receptors and subsequent initiation of postganglionic Na^+ spike. Atropine and the ω-conotoxins produced a similar degree of inhibition against DMPP contractures. However, the inhibition was never complete (about 50–70%) and inversely related to the concentration of DMPP applied (Table 1). Combinations of ω-conotoxins inhibited the contracture to the similar extent. ω-Agatoxin IVA (300 nM) did not affect the DMPP-induced contracture either alone or in the presence of ω-conotoxins or atropine (not shown).

Effects of ω-conotoxins on acetylcholine contracture

Acetylcholine (1–3 μM, 60 s) induced a tonic contracture, the amplitude of which was similar to that of the phasic contracture induced by DMPP. In contrast to the DMPP contracture, the acetylcholine contracture was inhibited neither by tetrodotoxin (3 μM) nor by tubocurarine (10 μM) whereas it was abolished by atropine (0.1 μM). ω-Conotoxins, at concentrations (100–3000 nM) high enough to abolish field stimulation-induced twitch responses, did not affect acetylcholine contracture (not shown).

Discussion

At low frequency stimulation, all four ω-conopeptides abolished the neuromuscular transmission in the guinea-pig ileum

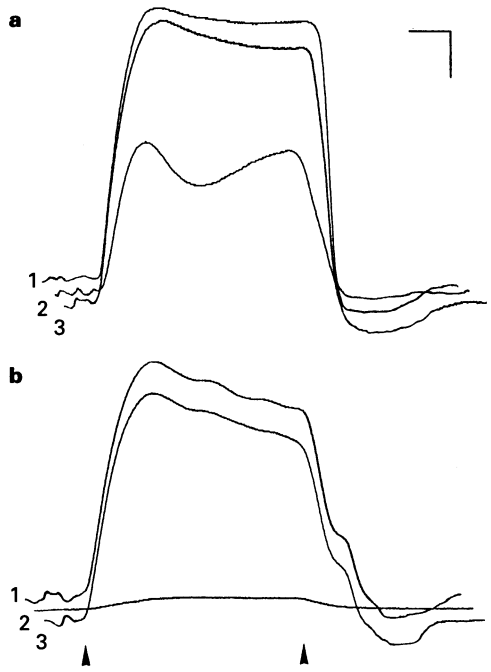

Figure 5 Inhibition of tetanic contracture by ω-conotoxin MVIIC and Ni^{2+}: ileum preparations were stimulated with trains of pulses (30 Hz for 5 s in between arrow-heads). Tetanic contractures were evoked (**1**) before, (**2**) 20 min after a pretreatment with either ω-conotoxin MVIIC (300 nM, a) or Ni^{2+} (5 mM, b), and (**3**) 20 min after washout of the Ca^{2+} channel blocker. Calibrations: 1 g and 1 s.

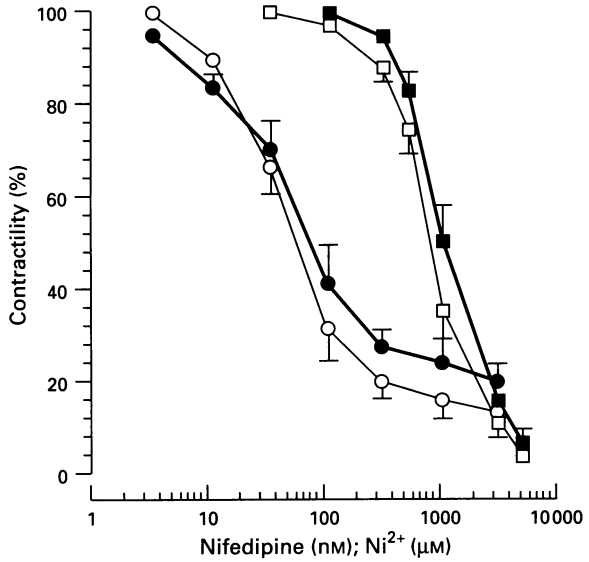

Figure 6 Concentration-dependent inhibition of twitch response and contracture by Ni^{2+} and nifedipine: ileum preparations were stimulated with single (0.1 Hz, ○, □) or trains of pulses (30 Hz for 5 s every 15 min, ●, ■). Nifedipine (○, ●) and Ni^{2+} (□, ■) were applied cumulatively. The effects of Ca^{2+} channel blockers at each concentration were observed for 20 min from which the maximal inhibitions of twitch response or contracture area were plotted. $n=4$.

and the effects were inversely related to the extracellular Ca^{2+} concentration. The inhibitions were not due to any alteration of muscarinic receptor as judged from the unchanged acetylcholine contracture after ω-conotoxins. Hence, the inhibitory effects of the ω-conotoxins on the ileum contraction can be regarded as mediated by impairment of the release of acetylcholine. The observed toxin-induced transient facilitation of the contractile response could have resulted from a preferential inhibition of the field stimulation-evoked release of inhibitory transmitters. In guinea-pig ileum, antagonists of adenosine (8-phenyltheophylline), noradrenaline (phentolamine) and Leu/Met-enkephalins (naloxone) produced 10–30% enhancement of twitch responses (unpublished), suggesting that field stimulation excites the inhibitory neurones as well.

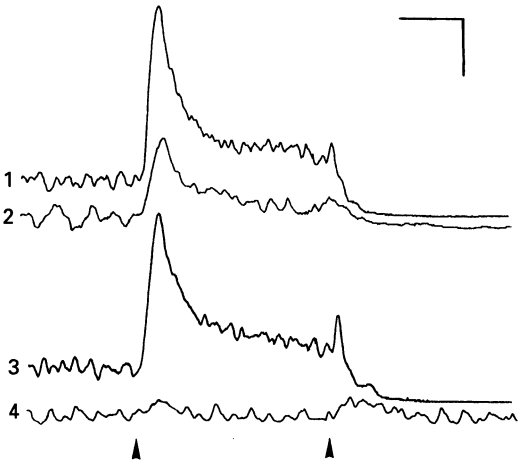

Figure 7 Inhibition of DMPP-induced contracture by tetrodotoxin and ω-conotoxins: the ileum preparation was contracted with DMPP (30 μM for 30 s in between arrow-heads). DMPP-contractures were evoked (**1**) before; (**2**) 20 min after a treatment with ω-conotoxins MVIIA (300 nM) plus MVIIC (3000 nM); (**3**) after washout of ω-conotoxins and (**4**) 20 min after a treatment with tetrodotoxin (1 μM). Calibrations: 1 g and 10 s.

N-channel predominates at low frequency stimulation

The inhibitory effects of the four ω-conopeptides, in general, were qualitatively similar to each other but differed in potency (MVIIA > GVIA > SVIB > MVIIC) and reversibility (MVIIC was readily reversible while GVIA was the least reversible). The inhibitory effects of ω-conotoxins GVIA and MVIIC were qualitatively in line with those reported by Boot (1994). ω-Conotoxins GVIA and MVIIA produced inhibition in the concentration-range that blocked the N-type Ca^{2+} channel (Olivera *et al.*, 1985; Valentino *et al.*, 1993). ω-Conotoxin MVIIC has been shown to block O-, P-, Q- as well as N-type channels (Zhang *et al.*, 1993). However, the estimated IC_{50} value of ω-conotoxin MVIIC was two orders of magnitude greater than that needed to inhibit the O-type channel (~nM, Adams *et al.*, 1993) and ω-agatoxin IVA, which inhibits P- as well as Q-type channels (Sather *et al.*, 1993; Wheeler *et al.*, 1994; Randall & Tsien, 1995), did not significantly affect the electrically induced response of the ileum. Binding studies suggest that ω-conotoxins MVIIA and GVIA share the same binding site on the N-type Ca^{2+} channel (Ramilo *et al.*, 1992; Kristipati *et al.*, 1994), while ω-conotoxin MVIIC targets additional distinct high affinity sites (Hillyard *et al.*, 1992; Wheeler *et al.*, 1994). The binding sites of ω-conotoxin SVIB are not yet fully delineated, but it undoubtedly blocks N-type channels and may bind to other classes of channel as well (Ramilo *et al.*, 1992; Fox, 1994). Whether the L- and T-type Ca^{2+} channel blockers, nifedipine and Ni^{2+}, also inhibited neurotransmission is difficult to assess because (1) the contraction of smooth muscle is intimately related to L-type channel activation (Droogmans & Callewaert, 1986) and (2) the two blockers also suppressed myogenic contractures induced by high K^+ and acetylcholine (not shown). Nevertheless, this possibility is not likely in view of the complete inhibition of twitch response by a variety of N-channel blockers. Indeed, L-type channel blockers did not modify [^{3}H]-

acetylcholine release from rat myenteric plexus (Wessler *et al.*, 1990) and Ni^{2+} at low concentrations (~100 μM) that selectively block the T-type Ca^{2+} channel (Ellinor *et al.*, 1993) did not produce persistent inhibition of twitch responses. Taken together, it appears that, in the guinea-pig myenteric plexus, the pharmacological properties of the Ca^{2+} channel responsible for acetylcholine release upon single stimulation relates to the N-type channel, rather than to L-, O-, P-, Q- or T-types.

In contrast to the myenteric plexus, the acetylcholine release from guinea-pig cardiac parasympathetic nerve was resistant to ω-conotoxin GVIA, while ω-conotoxin MVIIC was equipotent in both organs (Hong & Chang, 1995a). It was inferred that an OPQ-family Ca^{2+} channel participates in the acetylcholine release in the cardiac parasympathetic nerve. In the intestinal smooth muscle, stimulation of muscarinic receptors activate non-selective cation channels (Inoue & Isenberg, 1990), phosphatidylinositol hydrolysis (Komori & Bolton 1991) and the diacylglycerol cascade (Vivaudou *et al.*, 1988) to modulate intracellular Ca^{2+} levels and contractility. The incomplete inhibition of nerve stimulation-induced contraction after blockade of L-type Ca^{2+} channels suggest that these muscarinic receptor-linked pathways have physiological significance.

Multiple channel activation on high frequency and DMPP stimulation

Compared with the complete inhibition of twitch responses by tetrodotoxin, atropine and the ω-conotoxins, the tetanic contractures induced by high frequency stimulation, particularly at 30 Hz, were less inhibited by these agents. Moreover, the extent of maximal inhibition produced by these agents was different, being tetrodotoxin > atropine > MVIIC > GVIA, MVIIA and SVIB.

Field stimulation might, by bypassing activation of Na^+ channels produce a low level depolarization of nerve terminals. The depolarization, though insufficient to evoke excitation-secretion coupling with low frequency stimulation, could accumulate to a threshold resulting in a substantial Ca^{2+} influx which would modulate transmitter release at high frequency stimulation. The incomplete inhibition by tetrodotoxin under these conditions would support this possibility.

Furthermore, the mode of neurotransmitter release resulting from repetitive nerve terminal depolarization might be different from that induced by transient depolarization in the types (pools) of neurotransmitter released or the classes of Ca^{2+} channel activated (Gaur *et al.*, 1994; Regehr & Mintz, 1994; Wheeler *et al.*, 1994; Gonzalez Burgos *et al.*, 1995; Hong *et al.*, 1995). The lesser extent of block of tetanic contractures by atropine than by tetrodotoxin suggests that neurotransmitter(s) other than acetylcholine may be released. For example, the myenteric plexus accommodates many neuropeptides (*c.f.* Yau, 1989). The low degree inhibition of contractures by the ω-conotoxins compared with tetrodotoxin may suggest that other subtypes of Ca^{2+} channels could be activated on high frequency stimulation. The more efficacious suppression of tetanic contracture by ω-conotoxin MVIIC than by ω-conotoxins MVIIA, GVIA and SVIB is in line with the broader spectrum of action of ω-conotoxin MVIIC than the other ω-conotoxins (Hillyard *et al.*, 1992). However, the incomplete block of ω-conotoxin MVIIC suggests that Ca^{2+} channels other than N-, O-, P-, and Q-types could be recruited on high frequency stimulation. Recently, a residual Ca^{2+} current has been isolated in the cell body of cerebellar granule neurones after complete blockade of N-, O-, P- and Q-type channels (Randall & Tsien, 1995). This R-type current, like currents carried by T-type channels, inactivates rapidly and may modulate complex electrical activity of neurones rather than directly regulate neurotransmitter release (Ellinor *et al.*, 1993; Soong *et al.*, 1993). In dorsal raphe neurones, a non-OPQ- and non-R-type Ca^{2+} current, which shares many characteristics with those of the N-type-Ca^{2+} current but is resistant to ω-conotoxin GVIA, has also been described (Penington & Fox, 1995). The possibility remains that repetitive depolarization of nerve terminals might impose/accelerate the unblocking action of ω-conotoxins as the inhibitory activity might be dependent on membrane potential or the medium ionic activity (Hillyard *et al.*, 1992; Mintz *et al.*, 1992). It is also possible that the relative ineffectiveness of ω-conotoxins under high frequency stimulation may in part be due to a frequency-dependent facilitation of the Ca^{2+} current (Dolphin, 1996). However, both possibilities cannot adequately explain the reduction in the effectiveness of atropine/tetrodotoxin under the stimulation conditions.

The ileum contracture upon application of DMPP was completely blocked by tubocurarine and tetrodotoxin but was incompletely inhibited by atropine, suggesting that the excitation of smooth muscle is not due entirely to release of acetylcholine. Like high frequency stimulation, stimulation of the plexus by DMPP might trigger repetitive axonal action potential and release of neuropeptides, such as vasoactive intestinal polypeptide, which excites myenteric neurones (Williams & North, 1979; Grider & Jin, 1993). The same degree of inhibition of DMPP contractures by all the ω-conotoxins and by atropine suggested that ω-conotoxins are affecting only the cholinergic pathway. It may be inferred that the DMPP-induced release of acetylcholine is dependent on the activity of N-type channels whereas that of other excitatory transmitters is not.

In summary, in the myenteric plexus of guinea-pig ileum, the neuromuscular transmission under low frequency stimulation was inhibited by a variety of ω-conotoxins which all block N-type Ca^{2+} channels. It is not yet clear whether high frequency stimulation may recruit additional Ca^{2+} channels, mobilize intracellular Ca^{2+} stores, or secrete non-cholinergic excitants for the excitation-secretion coupling of the myenteric plexus.

This work was supported by a grant from the National Science Council (NSC86-2331).

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(Received November 10, 1995
Revised February 8, 1996
Accepted February 15, 1996)