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ω-Conotoxins block neurotransmission in the rat vas deferens by binding to different presynaptic sites on the N-type Ca^{2+} channel

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Abstract

Electrically-induced twitch responses of the prostatic segment of vas deferens (0.1 Hz, 65 V, 1 ms) are mainly due to the transient presynaptic release of ATP, which acts postsynaptically on non-adrenergic receptors to contract smooth muscle cells. These responses were fully blocked by nanomolar concentrations of the ω-conotoxins GVIA, MVIIA, and MVIIC, most likely by inhibiting Ca^{2+} entry through presynaptic N-type Ca^{2+} channels controlling the release of ATP. Repeated washout of the toxins allowed the recovery of contractions, except for ω-conotoxin GVIA, whose inhibitory effects remained unchanged for at least 60 min. In addition, micromolar concentrations of ω-conotoxin MVIIC were unable to protect against the irreversible inhibition of twitch contractions induced by nanomolar concentrations of ω-conotoxin GVIA. At low extracellular Ca^{2+} concentrations (1.5 mM), 20 nM of ω-conotoxin GVIA or MVIIA inhibited completely the twitch contractions in about 10 min. In 5 mM Ca^{2+} the blockade of twitch contractions after 10 min was 70% for both toxins. In 1.5 mM Ca^{2+} ω-conotoxin MVIIC (1 μM) inhibited completely the twitch contraction after 10 min. In 5 mM Ca^{2+} blockade developed very slowly and was very poor after 30 min, ω-conotoxin MVIIC depressed the response by only 20%. These results are compatible with the idea that the three ω-conotoxins block the purinergic neurotransmission of the vas deferens by acting on presynaptic N-type voltage-dependent Ca^{2+} channels. However, ω-conotoxin MVIIC seems to bind to sites different from those recognised by ω-conotoxin GVIA and MVIIA, which are markedly differentiated by their Ca^{2+} requirements for binding to their receptors.

Keywords: Ca^{2+} channel; ω-Conotoxin GVIA; ω-Conotoxin MVIIA; ω-Conotoxin MVIIC; ω-Agatoxin IVA; Vas deferens; Purinergic neurotransmission

1. Introduction

Polypeptide ligands, collectively referred to as ω-toxins, have been widely used during the last years to define various subtypes of voltage-dependent Ca^{2+} channels in different central and peripheral neurones (Olivera et al., 1994). Thus, ω-conotoxin GVIA, which is present in the venom of molluscs belonging to the genus *Conus*, is considered as a selective, irreversible blocker of N-type Ca^{2+} channels (McCleskey et al., 1987). Another less studied peptide, ω-conotoxin MVIIA, appears to bind to the same site as ω-conotoxin GVIA (Fox, 1995). Furthermore, low concentrations of the funnel-web spider toxin, ω-agatoxin IVA, irreversibly inhibit P-type Ca^{2+} channels (Mintz et al., 1992). In addition, a new toxin from the marine snail *Conus magus*, ω-conotoxin MVIIC, has been recently characterised (Hillyard et al., 1992). Though P- as well as N-type Ca^{2+} channels seem to recognise such a toxin, a novel additional component of the whole-cell Ca^{2+} current is also blocked by the ω-conotoxin MVIIC. This led to the belief that this toxin can identify a new subtype of neuronal Ca^{2+} channel named Q (Wheeler et al., 1994; López et al., 1994) or O (Adams et al., 1993).

The use of ω-toxins as diagnostic pharmacological tools

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to identify different Ca^{2+} channels meets with two obstacles: first, their limited selectivity and second, the interference of divalent cations with binding to their receptors. The limited selectivity is exemplified by ω-agatoxin IVA blocking P channels at nanomolar concentrations and Q channels at micromolar concentrations (Wheeler et al., 1994), and by ω-conotoxin MVIIC, which blocks N-, P- as well as Q-type Ca^{2+} channels (Hillyard et al., 1992; Wheeler et al., 1994; Randall and Tsien, 1995). An example of the second complicating obstacle is the attenuation of [^{125}I]ω-conotoxin GVIA binding to bovine adrenal medullary plasma membranes by increasing concentrations of divalent cations. Thus, Ca^{2+}, Sr^{2+} and Ba^{2+} prevent the binding of the toxin with IC_{50} values of 1, 1.2 and 1.8 mM, respectively (Ballesta et al., 1989).

Recently, Olivera et al. (1994) suggested an attractive hypothesis to explain the limited selectivity of ω-conotoxins to bind and block different Ca^{2+} channel subtypes. ω-Conotoxins and many spider toxins may be targeted to homologous ligand-binding pockets on different voltage-sensitive Ca^{2+} channel subtypes, the macrosites. The macrosite comprises multiple microsites that are potential points of focal contact with peptide ligands. In the case of the three known toxins that recognise N-type Ca^{2+} channels, they have common properties (i.e. blockade of such channels) but also some differences; for instance, the blockade is reversible (ω-conotoxin MVIIA) or irreversible (ω-conotoxin GVIA). To characterise their interactions at common sites, their effects at equilibrium must be studied. This requires long periods of exposure especially at the lower concentrations, which means that a test parameter is needed that remains constant for such long periods. This is not the case for the recording of whole-cell Ca^{2+} or Ba^{2+} currents through Ca^{2+} channels with voltage-clamp techniques as these currents decline with time (washout of the current; Fenwick et al., 1982). Radioligand binding experiments have also some limitations, especially when studying the reversibility of binding.

We have recently studied some toxin interactions at presynaptic sympathetic nerve terminals, using a sympathetic innervated organ, the guinea-pig left atrium. Measurement of the inotropic response was taken as an adequate index of the release of noradrenaline from electrically stimulated cardiac nerves, which is triggered by Ca^{2+} entry through N-type Ca^{2+} channels (Vega et al., 1995). However, the cardiac beat is greatly influenced by the extracellular Ca^{2+} concentration, and easily develops arrhythmias. Therefore, an alternative preparation was needed to perform the study on toxin interactions proposed here. A smooth muscle preparation with a less marked influence of the extracellular Ca^{2+} concentration on its contraction seemed to be more appropriate.

In the rat vas deferens, ω-conotoxin GVIA has been shown to cause an inhibition of neurotransmission in its prostatic part which is mostly non-adrenergic. This suggests the involvement of N-type Ca^{2+} channels in controlling the presynaptic release of neurotransmitter (Maggi et al., 1988). We therefore decided to perform the present study in such a preparation. The study was aimed at testing the hypothesis that the ω-conotoxins GVIA, MVIIA and MVIIC although blocking the N-type Ca^{2+} channel, do so by binding to different sites and likely, also through different molecular mechanisms.

2. Materials and methods

Male Wistar rats (300–400 g) were killed by a blow on the head. The vasa deferentia were removed and cleared of surrounding tissues. The prostatic half of the organ was placed in a 10-ml glass bath at 37°C, in Krebs-bicarbonate solution of the following composition (mM): NaCl 119, KCl 4.7, $MgSO_4$ 1.2, KH_2PO_4 1.2, $CaCl_2$ 1.8, $NaHCO_3$ 25, glucose 11, pH 7.4, bubbled with 95% O_2-5% CO_2. One gram of tension was applied and each tissue was allowed a 30-min initial equilibration period. Then, twitch contractions were recorded by means of an isometric transducer connected to an amplifier and recorder (Cibertec, Madrid, Spain). The contractions were generated by electrical field stimulation with pulses of 65 V, 0.5 ms duration and frequency of 0.1 Hz. Initially, some decay of the magnitude of contractions occurred, but soon they stabilised and remained constant for at least 90–120 min. That this contraction is neurogenic in nature was shown by the fact that tetrodotoxin (1 μM) abolished the twitches ($n = 3$). The fact that suramin (100 μM, $n = 3$) inhibited the responses by over 90%, strongly suggests that the neurotransmitter involved is ATP. The poor blockade produced by prazosin (0.3 μM, $n = 5$), even after a 45-min incubation, indicates a minor role for the release of noradrenaline from sympathetic nerve terminals in eliciting such twitch contractions under our experimental conditions. When a preparation responded with reproducible twitches, a given concentration of ω-toxin was added and the time course of its effects monitored. Usually, the toxin was then washed out to look for reversibility of its action. A single concentration of toxin was tested in each preparation, unless otherwise stated. The size of the initial contraction was measured in mm and mN, and was normalised to a percentage. The effects of a given concentration of toxin were then expressed as a percentage of the initial contraction. Results are presented as means ± S.E.M. Statistical differences between means were calculated by Student's *t*-test. *P* values smaller than 0.05 were taken as the level of significance.

ω-Agatoxin IVA was obtained from Peptide Institute (Osaka, Japan). The other three toxins were purchased from Bachem, UK. All other chemicals were reagent grade, either from Merck (Darmstadt, Germany) or Sigma (St. Louis, MO, USA).

3. Results

3.1. The effects of single concentrations of ω-conotoxins GVIA, MVIIC, MVIIA and ω-agatoxin IVA on twitch contractions of the vas deferens in response to electrical field stimulation

At a concentration of 100 nM, ω-conotoxin GVIA exerted an inhibition of twitch contractions, which were reduced by 50% after about 2 min, with a complete block occurring after less than 10 min. This inhibition was irreversible, since 60 min after washing out the toxin, the contractions were still fully suppressed (Fig. 1A). These data confirm previous reports on the potency and the irreversibility of the effects of ω-conotoxin GVIA (Maggi et al., 1988; De Lucca et al., 1990; Boot, 1994).

ω-Conotoxin MVIIC also produced a block of contractions, but a 30 times higher concentration than that for

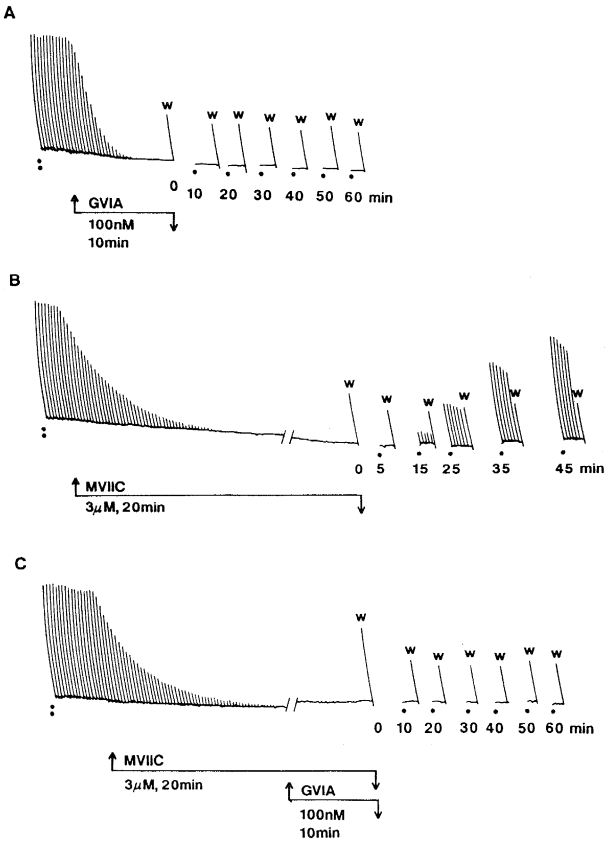

Fig. 1. (A) Typical experiment showing the time course of the effect of ω-conotoxin GVIA on twitch contractions of an electrically stimulated vas deferens (65 V, 0.5 ms, at 10-s intervals). The toxin was added (upward arrow) after the contractions stabilised (double dot) and was washed out after 10 min (downward arrow), starting the washout period (0 min). Note that records of the ensuing washout period did not detect any measurable contraction, which is consistent with the expected irreversibility of the effects of ω-conotoxin GVIA. Dots indicate the beginning of a stimulation period, and w indicates washout, with pause of stimulator and recorder. (B) Experiment in which ω-conotoxin MVIIC was used instead of GVIA. Note the time-dependent recovery. (C) Protection experiment in which ω-conotoxin MVIIC was incubated before and during incubation with ω-conotoxin GVIA. Note that the first ω-conotoxin incubation did not prevent the irreversible binding of ω-conotoxin GVIA with its receptor, since twitch responses were not obtained even after a 60-min washout.

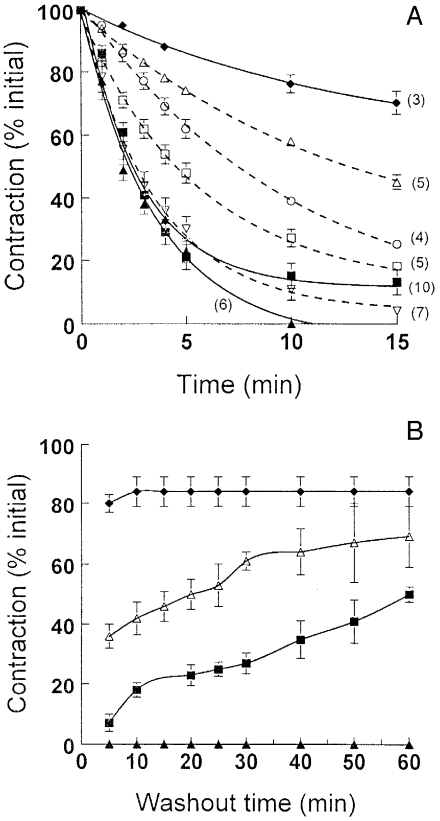

Fig. 2. (A) Time-course of blockade of electrically induced twitch contractions by ω-agatoxin IVA (100 nM, ♦), ω-conotoxin MVIIA (100 nM, ■), ω-conotoxin GVIA (100 nM, ▲) and four concentrations of ω-conotoxin MVIIC (100 nM, △, 0.3 μM, ○, 1.0 μM, □, and 3 μM, ▽). In (B) the time-course for recovery of contractions after washout of 100 nM (30 min) of the corresponding toxins is plotted. Data are means ± S.E.M. of the number of preparations shown in parentheses (A), or of 3–5 vasa deferentia in (B).

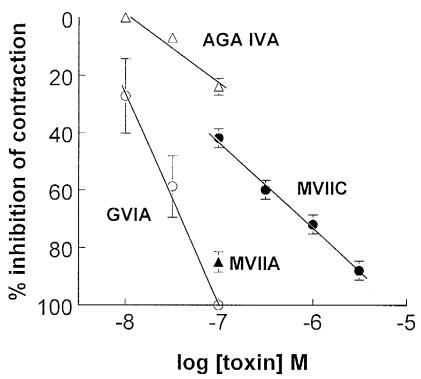

Fig. 3. Mean log concentration-effect lines for ω-agatoxin IVA, ω-conotoxin MVIIC and ω-conotoxin GVIA, obtained from experiments similar to that shown in Fig. 1, for a 10 min incubation period. The inhibition induced by a single concentration of ω-conotoxin MVIIA is also shown. Points are means ± S.E.M. of 3–10 experiments.

ω-conotoxin GVIA was necessary to induce a similar effect, within about 10 min. There was also a marked difference with ω-conotoxin GVIA, namely the reversibility of block. Though occurring slowly, repeated washout of ω-conotoxin MVIIC allowed a progressive recovery of contractions. About 70% of the initial contraction was observed after a 45-min washout period (Fig. 1B).

Since both toxins were described as interacting with N-type Ca^{2+} channels, protection experiments were performed. We used a dose-ratio ω-conotoxin MVIIC/ω-conotoxin GVIA that was 3 times higher than that used by Boot (1994), and a longer preincubation period (10 min instead of 5 min). In spite of these modifications, our results were similar to those of Boot (1994), since ω-conotoxin MVIIC could not prevent the irreversible block of contractions during the washout period due to the interaction with ω-conotoxin GVIA (Fig. 1C).

The results with a third toxin, ω-conotoxin MVIIA (100 nM), showed a similar degree of inhibition as that with ω-conotoxin GVIA, while a very weak inhibition was obtained with the same concentration of ω-conotoxin IVA (not shown).

3.2. The onset and offset of inhibition by the ω-toxins

Fig. 2A shows the effects of the maximal concentrations used of ω-conotoxins GVIA and MVIIA and of ω-agatoxin IVA, and of increasing concentrations of ω-conotoxin MVIIC on twitch contractions over a 15-min period. Fig. 2B shows the recovery of contractions after washout of 100 nM of the corresponding ω-toxins, over a 60-min period. A partial but substantial recovery was observed during this period for ω-conotoxin MVIIA and MVIIC. This was not the case for ω-conotoxin GVIA, whose inhibitory effects were still fully present. However, in two experiments the antagonism exerted by a lower concentration of ω-conotoxin GVIA (10 nM) was reversed by about 20% after 50 min (not shown). It is also noteworthy that, in spite of its small inhibitory effect, the influence of ω-agatoxin IVA was not reversed after washout.

3.3. Concentration-dependent effects of ω-toxins

Fig. 3 shows the regression lines for the inhibition of twitches by ω-conotoxin GVIA, ω-conotoxin MVIIC and ω-agatoxin IVA. The inhibition by a single concentration of ω-conotoxin MVIIA is also shown. Full suppression of the twitch responses was achieved with ω-conotoxin GVIA (IC_{50} ~ 20 nM) and ω-conotoxin MVIIC (IC_{50} ~ 200 nM). Up to a concentration known to block completely P-type Ca^{2+} channels (100 nM, Mintz et al., 1992) ω-agatoxin IVA caused only a 20% inhibition of twitch contractions. Two differences were observed in relation to previous reports: (a) the preparation was about 10 times

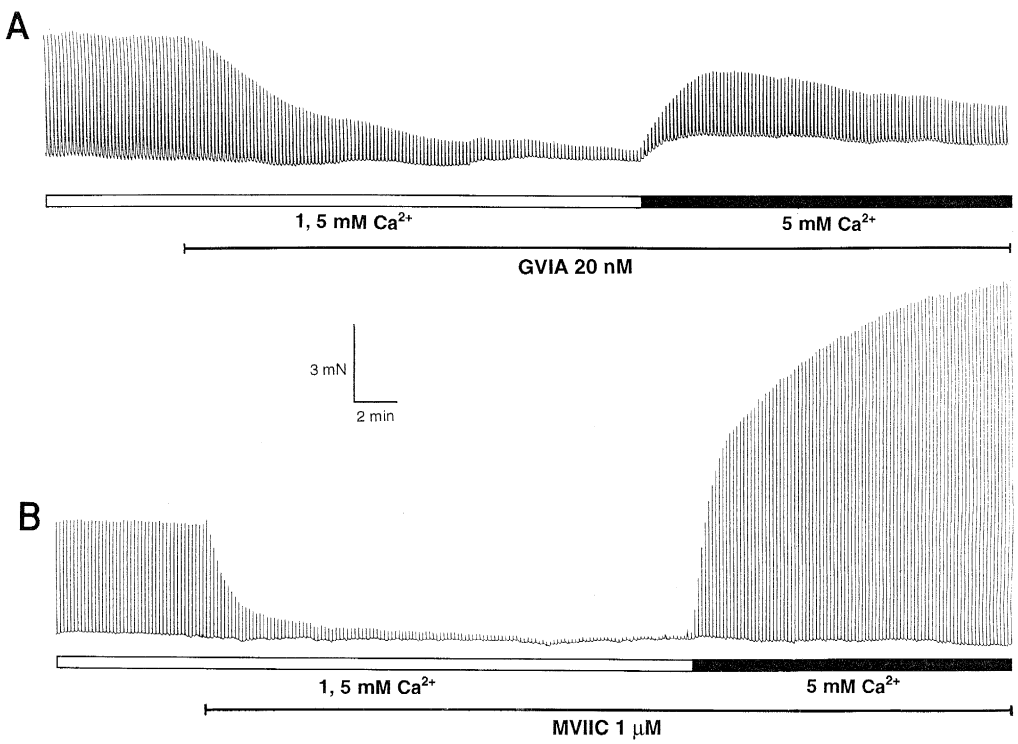

Fig. 4. Reversal by excess Ca^{2+} of the blockade of twitch contractions induced by ω-conotoxin GVIA (GVIA, panel A) or ω-conotoxin MVIIC (MVIIC, panel B). Contractions were electrically induced first with the preparation bathed in Krebs solution containing 1.5 mM Ca^{2+} (white horizontal bar) or 5 mM Ca^{2+} (black horizontal bar). The toxins were added at the indicated concentrations during the period shown by the horizontal line at the bottom of each record.

more sensitive to ω-conotoxin MVIIC than in the experiments described by Boot (1994), using the whole vas deferens; (b) the twitch contractions were partly inhibited by ω-agatoxin IVA, an effect that could not be detected by Lundy and Frew (1994).

3.4. Reversal by excess Ca^{2+} of the blocking effects of ω-conotoxins

To define whether the concentration of Ca^{2+} ions affected the blocking effects of the toxins, the experiment shown in Fig. 4 was performed. This figure shows the original traces obtained from two vasa deferentia initially incubated in low Ca^{2+} solution (1.5 mM Ca^{2+}) and subsequently in 5 mM Ca^{2+}. The initial twitch contractions induced by electrical stimulation were similar in both preparations. The concentrations of toxins selected (20 nM for ω-conotoxin MVIIC) were 'submaximal' to block the contractions in 1.8 mM Ca^{2+} (see Fig. 3).

Without delay, the contractions obtained in 1.5 mM Ca^{2+} started to decline, to reach less than 10% of their initial size 10 min thereafter. The contraction was halved in 2.5 min in the case of ω-conotoxin GVIA (mean of 4 preparations), and 1.6 min in the case of ω-conotoxin MVIIC (mean of 4 preparations). The contractions were almost totally inhibited in about 10–15 min.

The increase in the $[Ca^{2+}]$ from 1.5 to 5 mM partially restored the contractile response in the preparation treated with ω-conotoxin GVIA ($19.8 \pm 8.2\%$; mean ± S.E.M. of 3 experiments). In the case of the preparation treated with ω-conotoxin MVIIC, the contraction recovered very rapidly when the Ca^{2+} concentration was increased from 1.5 to 5 mM. In 3 experiments, the contraction reached an amplitude of 170.3 ± 24 mm, about 40% higher than the contraction obtained in 5 mM Ca^{2+} in the absence of toxin ($P < 0.025$).

The contractile response triggered by 100 μM of exogenous ATP was not significantly different in 1.5 mM or 5 mM Ca^{2+}. Thus, the contraction amounted to 20.5 ± 3.4 mm in 1.5 mM Ca^{2+} and 20.3 ± 2.5 mm in 5 mM Ca^{2+} ($n = 4$). This, together with the poor recovery of contraction in the ω-conotoxin GVIA-treated tissue, strongly suggests that the changes in the contractions seen at low and high $[Ca^{2+}]$ have a presynaptic origin.

3.5. Blockade of twitch contractions by ω-toxins at low and high Ca^{2+}

In these experiments, the effects of a single concentration of toxins on twitch contractions were explored at low (1.5 mM) and high concentrations of extracellular Ca^{2+} (5 mM). In 1.5 mM Ca^{2+}, ω-conotoxin GVIA (20 nM) produced a progressive decline of the contractile responses, which were fully suppressed 10 min after addition of the toxin. The amplitude of the response was halved in 2.5 min. In 5 mM Ca^{2+}, the rate of blockade was delayed (half blockade took place in 6.3 min) and the inhibition of contraction after 10 min of toxin incubation was 70%. The blockade progressed to 90% after 40 min of incubation with ω-conotoxin GVIA (Fig. 5A).

ω-Conotoxin MVIIA (20 nM) behaved similarly to ω-conotoxin GVIA in low and high Ca^{2+} solutions. In 1.5 mM Ca^{2+}, the blockade was almost complete and developed faster (50% inhibition in 2.2 min). In high Ca^{2+}, the inhibition of twitch contractions weas also delayed (50% blockade in 3.2 min); about 20% of the responses remained unblocked after 25 min of toxin incubation (Fig. 5B).

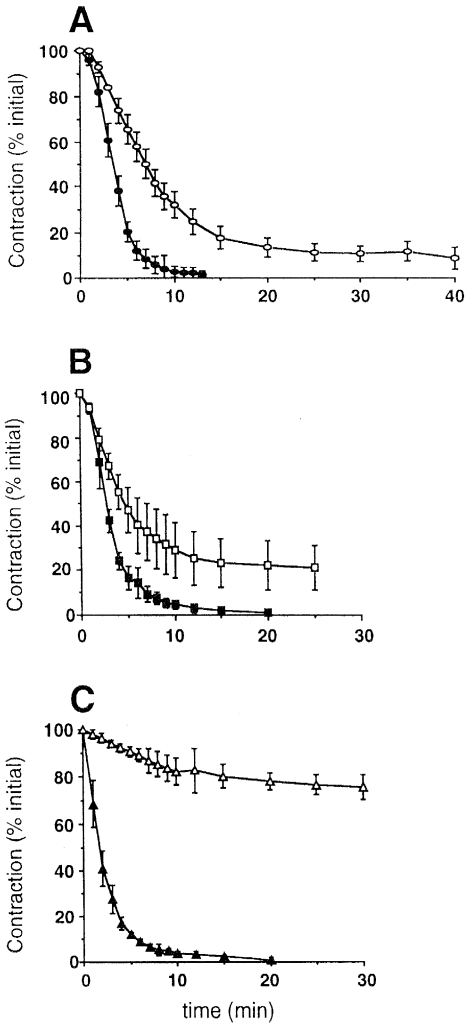

Fig. 5. Effects of Ca^{2+} ions on the effects of ω-conotoxin GVIA (panel A, 20 nM), ω-conotoxin MVIIA (panel B, 20 nM), and ω-conotoxin MVIIC (panel C, 1 μM) on twitch contractions of the vas deferens induced by electrical field stimulation. The preparations were initially bathed with Krebs solution containing either 1.5 mM Ca^{2+} (closed symbols) or 5 mM Ca^{2+} (open symbols), and they were stimulated continuously at 0.1 Hz. Once the twitch contractions stabilised, the toxins were added (time 0 at the abscissa) and the time course of their blocking effects on twitch contractions was monitored during the time periods shown on the abscissae. Data were normalised as percentages of the initial contraction; they are means ± S.E.M. of 4 preparations for each toxin and Ca^{2+} concentration.

Finally, the behaviour of ω-conotoxin MVIIC (1 μM) considerably differed from that of ω-conotoxin GVIA and MVIIA (Fig. 5C). In low Ca^{2+} (1.5 mM), the blockade was even faster, since the contractions were halved in 1.6 min. Full inhibition of contractions occurred after 10 min of toxin incubation. However, in high Ca^{2+} (5 mM), the blockade developed very slowly and was very poor. In fact, after 30 min of incubation with ω-conotoxin MVIIC as much as 80% of the contractile response remained unblocked.

4. Discussion

The results of this investigation confirm those of previous reports attributing a dominant role to the N-subtype of voltage-dependent Ca^{2+} channels in controlling the release of neurotransmitter responsible for the twitch contractions of the rat vas deferens in response to electrical field stimulation. This conclusion is based on the potent, irreversible blockade of such contractions by ω-conotoxin GVIA, a specific blocker of neuronal N-type Ca^{2+} channels (Olivera et al., 1994), added to the effect of ω-conotoxin MVIIA. The effect of ω-conotoxin MVIIA has not been previously described in vas deferens. Contrary to the expectations from previous binding experiments with neuroblastoma cells (Fox, 1995), but not with neocortical membranes (Stoehr and Dooley, 1993), the effect of this toxin was reversible, since a partial recovery was observed after 45 min (Fig. 2B). In contrast to the agreement on the interpretation of the effects of ω-conotoxin GVIA and MVIIA, as being due to an interaction with N-type channels, some difficulties arise with the interpretation of the behaviour of ω-conotoxin MVIIC and ω-agatoxin IVA.

ω-Conotoxin MVIIC has been used to characterise voltage-dependent Ca^{2+} channel subtypes, and has been shown to be a non-selective blocker of various neuronal Ca^{2+} channel subtypes. For instance, in bovine chromaffin cells this toxin inhibits the N as well as the P components of the whole-cell Ba^{2+} currents. Furthermore, it blocks an additional component which is resistant to ω-conotoxin GVIA and to ω-agatoxin IVA and which is likely associated with a Q-type Ca^{2+} channel (López et al., 1994). It is important to point out that blockade by ω-conotoxin MVIIC of the whole-cell current flowing through Ca^{2+} channels develops very slowly and strongly depends on the divalent cation concentration (Albillos et al., 1996). This slow blockade has also been seen in cerebellar granular cells, where ω-conotoxin MVIIC blocks half of the whole-cell Ba^{2+} current (Randall and Tsien, 1995). This is in line with the results obtained here in the rat vas deferens, showing a slowly developing blockade of twitch contractions, mainly at lower concentrations. This slow effect of ω-conotoxin MVIIC agrees with its slow equilibration with target receptors in radioligand binding experiments performed with rat brain membranes (Hillyard et al., 1992). Under our experimental conditions, we observed that a steady state was not attained after 10 min neither with 100 nM nor with a concentration 10 times higher. These characteristics of the toxin could explain the poor blockade by ω-conotoxin MVIIC of twitch contractions of the rat vas deferens observed by Boot (1994). As shown in Fig. 3, a 50% blockade of contractions by ω-conotoxin MVIIC was only achieved at concentrations higher than 100 nM. This and even higher concentrations of the toxin are routinely being used in different recent studies (López et al., 1994; Randall and Tsien, 1995; Magnelli et al., 1995).

These experiments with ω-conotoxin MVIIC might raise some doubts about N-type channels being the only source of neuronal Ca^{2+} controlling the neurogenic responses. Thus, 1–5 μM of the toxin blocks the Q channels in bovine chromaffin cells (López et al., 1994) and in cerebellar granule cells (Randall and Tsien, 1995). But at these concentrations ω-conotoxin MVIIC also targets the P- and N-type channels in chromaffin cells (López et al., 1994) and in central neurons (Hillyard et al., 1992). The P channel is blocked slowly and irreversibly and the N channel quickly and reversibly (Swartz et al., 1993). This might explain the partial block induced by the P channel blocker ω-agatoxin IVA (Fig. 2A). The lack of protection by ω-conotoxin MVIIC against the irreversible blockade of ω-conotoxin GVIA (Fig. 1C) is in line with the conclusion that other types or subtypes of Ca^{2+} channels might control the non-adrenergic neurogenic twitch responses of the rat vas deferens. The possibility also exists that in blocking the N-channel, ω-conotoxin MVIIC binds to a site different from that used by ω-conotoxin GVIA. This belief is strengthened by the results on the interactions of the toxins with Ca^{2+} ions.

Profound variations of the binding of ω-conotoxins to membranes have been associated with changes in the concentrations of divalent cations (Ballesta et al., 1989; Stoehr and Dooley, 1993; Wagner et al., 1988; Witcher et al., 1993). These biochemical findings have functional correlates. Thus, the blockade by ω-conotoxin GVIA of the whole-cell Ba^{2+} current in voltage-clamped bovine chromaffin cells is delayed in the presence of 10 mM Ba^{2+}, compared with 2 mM Ba^{2+}. This difference is even more striking in the case of ω-conotoxin MVIIC, since the inhibition of I_{Ba} in 10 mM Ba^{2+} was largely prevented. A similar picture emerges from the present study. In low Ca^{2+} (1.5 mM) the blockade of twitch contractions by ω-conotoxin MVIIC was much faster and more pronounced than in 5 mM Ca^{2+}. In fact, at the higher concentration of Ca^{2+} the inhibition was slight. This contrasts with the smaller influence that Ca^{2+} ions exert on the blocking effects of ω-conotoxin GVIA and MVIIA.

A puzzling question is the different behaviour of ω-conotoxin MVIIC in the guinea-pig atrium and in the rat vas deferens. While in the guinea pig the toxin protected fully against the irreversible blockade of neurotransmission induced by ω-conotoxin MVIIA (Vega et al., 1995), ω-

conotoxin MVIIC was incapable of affording any protection against the irreversible blockade of the rat vas deferens neurotransmission induced by ω-conotoxin GVIA (present study). There were no major differences in toxin concentrations, exposure time periods, nutrient solutions or other experimental details. Thus the difference might reside in the nature of the binding site for ω-conotoxin GVIA in the heart sympathetic nerve, which might structurally differ from its binding site in 'purinergic' nerve terminals in the rat vas deferens. Whether these differences reflect the existence of subtypes of N channels controlling the release of noradrenaline (heart) or ATP (vas deferens) is a question whose answer requires direct access to nerve terminals, which is not possible with currently available technology.

In conclusion, the binding to and blockade of N-type Ca^{2+} channels of the purinergic nerve terminals of the rat vas deferens by ω-toxins GVIA, MVIIA and MVIIC are strongly delayed by excess Ca^{2+} concentrations. This is particularly true for ω-conotoxin MVIIC, which in addition did not protect against the irreversible blockade by ω-conotoxin GVIA, thus suggesting different binding sites for both toxins at the N channel.

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