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Effects of Ca^{2+} channel blocker neurotoxins on transmitter release and presynaptic currents at the mouse neuromuscular junction

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1 The effects of the voltage-dependent calcium channel (VDCC) blockers ω-agatoxin IVA (ω-AgaIVA), ω-conotoxin GVIA (ω-CgTx), ω-conotoxin MVIIC (ω-MVIIC) and ω-conotoxin MVIID (ω-MVIID) were evaluated on transmitter release in the mouse diaphragm preparation. The effects of ω-AgaIVA and ω-MVIIC were also evaluated on the perineurial calcium and calcium-dependent potassium currents, I_{Ca} and $I_{K(Ca)}$, respectively, in the mouse levator auris preparation.

2 The P- and Q-type VDCC blocker ω-AgaIVA (100 nM) and P- Q- and N-type channel blockers ω-MVIIC (1 μM) and ω-MVIID (3 μM) strongly reduced transmitter release (>80–90% blockade) whereas the selective N-type channel blocker ω-CgTx (5 μM) was ineffective.

3 The process of release was much more sensitive to ω-MVIIC (IC_{50} = 39 nM) than to ω-MVIID (IC_{50} = 1.4 μM). After almost completely blocking transmitter release (quantal content ~0.3% of its control value) with 3 μM ω-MVIIC, elevating the external [Ca^{2+}] from 2 to 10 mM induced an increase of ~20 fold on the quantal content of the endplate potential (e.p.p.) (from 0.2±0.04 to 4.8±1.4).

4 Nerve-evoked transmitter release in a low Ca^{2+}-high Mg^{2+} medium (low release probability, quantal content = 2±0.1) had the same sensitivity to ω-AgaIVA (IC_{50} = 16.8 nM) as that in normal saline solutions. In addition, K^+-evoked transmitter release was also highly sensitive to the action of this toxin (IC_{50} = 11.5 nM; 100 nM >95% blockade). The action of ω-AgaIVA on transmitter release could be reversed by toxin washout if the experiments were carried out at 31–33°C. Conversely, the effect of ω-AgaIVA persisted even after two hours of toxin washout at room temperature.

5 Both the calcium and calcium-dependent potassium presynaptic currents, I_{Ca} and $I_{K(Ca)}$, respectively, were highly sensitive to low concentrations (10–30 nM) of ω-AgaIVA. The I_{Ca} and the $I_{K(Ca)}$ were also strongly reduced by 1 μM ω-MVIIC. The most marked difference between the action of these two toxins was the long incubation times required to achieve maximal effects with ω-MVIIC.

6 In summary these results provide more evidence that synaptic transmission at the mammalian neuromuscular junction is mediated by Ca^{2+} entry through P- and/or Q-type calcium channels.

Keywords: ω-Agatoxin IVA; ω-conotoxin GVIA; ω-conotoxin MVIIC; ω-conotoxin MVIID; calcium channel blockers; transmitter release; calcium channels; synaptic transmission; presynaptic currents; neuromuscular junction

Introduction

Voltage-dependent calcium channels (VDCC) play a key role in neuronal signalling as mediators of Ca^{2+} entry during the process of transmitter release (Katz, 1969; Llinás *et al*., 1976; Augustine & Charlton, 1986). Biophysical and pharmacological studies together with molecular cloning strategies have led to the identification of various VDCC types (Nowycky *et al*., 1985; Bean, 1989; Llinás *et al*., 1992; Zhang *et al*., 1993). Toxins extracted from the venom of predator species whose strategy involves paralysing their prey, like the fish-hunting snails and insect-hunting spiders, have been found to target selectively many of these VDCC (Olivera *et al*., 1994). These natural toxins, together with their synthetic counterparts and molecular variants, have become invaluable tools in the identification of the role of different VDCC in central and peripheral synaptic transmission in many species (see Olivera *et al*., 1994; Dunlap *et al*., 1995). Based on biophysical and pharmacological characteristics, VDCC have been classified into the T-, L-, N-, P-, Q- and R-types (Nowycky *et al*., 1985; Bean, 1989; Llinás *et al*., 1992; Zhang *et al*., 1993). The L-, N-, P- and Q-type channels, all of which require high voltages to activate, have been shown to participate in the process of release in several systems. L-type channels, can be identified by their high sensitivity to dihydropyridines while the N-, P- and Q-type channels, which are all insensitive to dihydropyridines, can be further discriminated based on their differential sensitivity to neurotoxins (Olivera *et al*, 1994; Dunlap *et al*., 1995). N-type channels are specifically targeted by ω-conotoxin GVIA (ω-CgTx), a polypeptide purified from the venom of the marine snail *Conus geographus* (Olivera *et al*., 1984). P-type channels, are strongly antagonized by the funnel-web spider toxin (FTX) and by ω-agatoxin IVA (ω-AgaIVA), a low molecular weight fraction and a polypeptide, respectively, both purified from the venom of the funnel-web spider *Agelenopsis aperta* (Llinás *et al*., 1989; Mintz *et al*., 1992a,b). Q-type channels, which are closely related to the P-type channels from the molecular point of view (Stea *et al*., 1994; Westenbroek *et al*., 1995), are also blocked by ω-AgaIVA. However, they can be discriminated from the P-type channels by their lower sensitivity to this toxin and by their much higher degree of inactivation (Zhang *et al*., 1993; Randall & Tsien, 1995). Two other conotoxins which target P-, Q- and N-type channels, ω-conotoxin MVIIC (ω-MVIIC) and ω-conotoxin MVIID (ω-MVIID), have been identified from cDNA libraries made from

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the venom duct of the marine snail *Conus magus*. These two synthetic toxins were found to bind with high affinity to both the 'N-type' and the 'non N-type' sites in rat brain membranes (Hillyard *et al*., 1992; Monje *et al*., 1993) and to inhibit K^+-evoked Ca^{2+} uptake from rat brain synaptosomes (Hillyard *et al*., 1992; Monje *et al*., 1993; Alvarez-Maubecin *et al*., 1995). In addition, ω-MVIIC has been shown to be a potent blocker of synaptic transmission both at the central (Wheeler *et al*., 1994a) and peripheral nervous system (Bowersox *et al*., 1995; Sugiura *et al*., 1995; Lin & Lin Shiau, 1995; Wright & Angus, 1996). The effects of ω-MVIID on synaptic transmission have not been studied so far. At the mature mammalian neuromuscular junction, VDCC of the P-type family (P/Q) seem to be the only VDCC involved in nerve-evoked transmitter release. This assumption is based on the inhibitory effects of FTX (Uchitel *et al*., 1992), ω-AgaIVA (Protti & Uchitel, 1993; Hong & Chang, 1995; Bowersox *et al*, 1995; Protti *et al*., 1996; Wright & Angus, 1996; Katz *et al*., 1996) and ω-MVIIC (Bowersox *et al*., 1995; Sugiura *et al*., 1995; Wright & Angus, 1996 and on the lack of effects of the N- and L-type channel blockers, ω-CgTx (Anderson & Harvey, 1987; Sano *et al*., 1987; Protti *et al*., 1991; Wright & Angus, 1996; but see Rossoni *et al*., 1994) and dihydropyridines (Penner & Dryer, 1986; Atchinson, 1989; Katz *et al*., 1996), respectively. In order to characterize further the type/s of VDCC involved in neuromuscular transmission in mammals, we studied the effects of ω-CgTx, ω-MVIIC, ω-MVIID and ω-AgaIVA on transmitter release at the mouse neuromuscular junction. The actions of ω-AgaIVA and ω-MVIIC were also evaluated on presynaptic currents in order to correlate the pharmacological profile of transmitter release with that of VDCC present at these motor terminals.

Methods

Experiments were carried out on either the diaphragm (for studying transmitter release) or the levator auris longus (for studying perineurial currents) muscles of adult male Swiss mice weighing 20–30 g. Animals were anaesthetized with an overdose of 2% tribromoethanol (0.3–0.5 ml 10 g^{-1} body wt; i.p.) and immediately exsanguinated. The corresponding muscle with its nerve supply was excised and dissected on a Sylgard-coated Petri dish containing a physiological saline solution (normal saline) of the following composition (mM): NaCl 137, KCl 5, $CaCl_2$ 2, $MgSO_4$ 1, $NaHCO_3$ 12, Na_2HPO_4 1 and glucose 11, continuously bubbled with 95% O_2/5% CO_2; pH = 7.1–7.2. The preparation was then transferred to the recording chamber to which the different working solutions and drugs were applied. Experiments were performed at room temperature (19–23°C) unless otherwise indicated. In some experiments the temperature in the recording chamber was controlled ($\pm$1°C) throughout the experiment by means of Peltier thermoelectric devices.

Evoked endplate potentials (e.p.ps) and miniature endplate potentials (m.e.p.ps) were recorded intracellularly with conventional glass microelectrodes filled with 3 M KCl (10–15 MΩ resistance). The mean quantal content of the evoked response in normal saline solution was estimated by the coefficient of variation method (see Martin, 1966; Hubbard *et al*., 1969). The mean quantal content (m) was calculated as:

$$m = (X_{epp})^2/((s.d._{epp})^2 - (s.d._{noise})^2)$$

where X is the mean amplitude and $s.d._{epp}$ and $s.d._{noise}$ are the standard deviations of the recorded e.p.p. amplitudes and of the noise, respectively.

Muscle contraction was prevented by 1.2–1.8 μM (+)-tubocurarine ((+)-Tc). After impalement of a muscle fibre, the nerve was continuously stimulated for 1 min at 0.5 Hz and then 50–100 successive e.p.ps were recorded. Records were rejected if the membrane potential, V_m, was < -60 mV or fell more than 5 mV during the recording period or if the 10–90% e.p.p. rise time was >1 ms. No corrections for non-linear summation were made, as (+)-Tc concentration was adjusted to obtain e.p.ps of less than 4 mV (McLachlan & Martin, 1981).

For evaluating transmitter release in low Ca^{2+}-high Mg^{2+} solutions the muscles were incubated in a saline solution of the following composition (mM): NaCl 137, KCl 5, $CaCl_2$ 0.9, $MgSO_4$ 6, $NaHPO_3$ 12, Na_2HPO_4 1 and glucose 11, continuously bubbled with 95% O_2/5% CO_2; pH = 7.1–7.2. The mean quantal content (m) was evaluated by the failures method (see Martin, 1966; Hubbard *et al*., 1969).

$$m = \ln (N/n_0),$$

where N is the total number of successive trials (100 at 0.5 Hz) and n_0 is the number of trials in which the response fails (absence of e.p.p.)

Spontaneous m.e.p.p. frequency (m.e.p.p.) was evaluated in normal saline solution and K^+-evoked fm.e.p.p. was always evaluated after 30 min of incubating the muscle with a saline solution of the following composition (mM): NaCl 137, KCl 10, $CaCl_2$ 2, $MgSO_4$ 1, $NaHCO_3$ 12, Na_2HPO 1 and glucose 11; continuously bubbled with 95% O_2/5% CO_2; pH = 71.–7.2. M.e.p.ps were recorded for periods of 1–2 min and their frequency of appearance counted directly on the oscilloscope screen.

Presynaptic currents were recorded by means of glass microelectrodes filled with 2 M NaCl (5–10 MΩ resistance) inserted, under visual control, into the perineurial sheath of small diameter nerve bundles (Mallart, 1985a). The passive-propagating signals recorded at this location in the perineurium are not exactly 'membrane currents' but they are closely related to membrane conductance changes generated both at the terminal nodes of Ranvier and at the synaptic terminals upon stimulation of the nerve. At this recording site, the currents generated at the nerve terminals are picked up with reversed polarity, so outward at the terminals will be referred to as inward and *vice versa*. In all cases upward deflections signal positivity at the recording electrode. In all the experiments, control and toxin treated records were obtained without changing the position of the electrode and once a stable recording had been achieved. The first downward deflection (Na^+ signal) was used as a control of signal stability throughout the experiment (for a description of the different components of these currents see Brigant & Mallart, 1982; Mallart, 1985a; Penner & Dryer, 1986). The muscles were incubated in normal saline solution in the presence of 30–50 μM (+)-Tc in order to avoid any postsynaptic contribution to the records. The I_{Ca} and $I_{K(Ca)}$ were obtained by adding 10 mM tetraethylammonium (TEA) and 250 μM 3,4-diaminopyridine (DAP) or 1 mM DAP, respectively, to the normal saline solution. Procaine (50–200 μM) was added, when necessary, to prevent repetitive firings.

The nerves were stimulated by using suction electrodes coupled to a pulse generator with an associated stimulus isolation unit. The recording electrodes were connected to an Axoclamp 2A amplifier (Axon Instruments). A distant Ag-AgCl electrode connected to the bath solution via an Agar bridge (Agar 3.5% in 137 mM NaCl) was used as reference. The signals were digitized (TL-1 DMA interface; Axon Instruments), stored and analysed by computer.

Values are expressed as mean $\pm$ s.e.mean. Statistical significance (P values in the text and figure legends) was evaluated by the two tailed Student's t test for unpaired values. Data points in the concentration-response curves were fitted by a sigmoidal concentration-response curve of the type: $y = 100/(1 + (10^{\hat{}(x - \log IC_{50})} n_H))$; where y = % of control value in the presence of the VDCC antagonist and x = log[VDCC antagonist]; n_H = Hill coefficient.

Chemicals

Tribromoethanol was purchased from Aldrich (Milwaukee, WI, U.S.A.). Bovine serum albumin (BSA), (+)-tubocurarine ((+)-Tc) and all other salts and reagents used were of analy-

tical grade and purchased from Sigma (St. Louis, MO, U.S.A.). The synthetic polypeptide ω-conotoxin GVIA (ω-CgTx) was purchased from Peptides International Co. (Louisville, KY, U.S.A.). The synthetic polypeptide ω-agatoxin IVA (ω-AgaIVA) was a generous gift from Dr N. Saccomano (Pfizer Inc., Grotton, CT, U.S.A.). The synthetic polypeptide ω-conotoxin MVIIC was purchased from RBI (Natick, MA, U.S.A.). The synthetic polypeptide ω-conotoxin MVIID was a generous gift from Dr Baldomero Olivera. In all cases, both control and toxin-treated fibres were assayed in the presence of 0.01% BSA.

Results

Effect of ω-agatoxin and ω-conotoxins on nerve-evoked transmitter release

As shown by the effects of the toxins on the estimated quantal content (Figure 1a), nerve-evoked transmitter release could be almost completely abolished by the P- and Q-type VDCC antagonist (ω-AgaIVA) and by the P-, Q- and N-type antagonists (ω-MVIIC and ω-MVIID) but was insensitive to the action of the selective N-type antagonist (ω-CgTx). The strong inhibitory effect of ω-AgaIVA and ω-MVIIC as well as the lack of effect of ω-CgTx are in agreement with previous results at the mouse neuromuscular junction (Sano *et al*., 1987; Protti *et al*., 1991; Protti & Uchitel, 1993; Bowersox *et al*., 1995; Hong & Chang, 1995; Wright & Angus, 1996; Katz *et al*., 1996). The three toxins that target P- and Q-type VDCC greatly reduced EPP amplitudes and increased their variance (Figure 1b). None of the toxins tested affected the resting membrane potential of the muscle fibres which ranged between −60 and −80 mV.

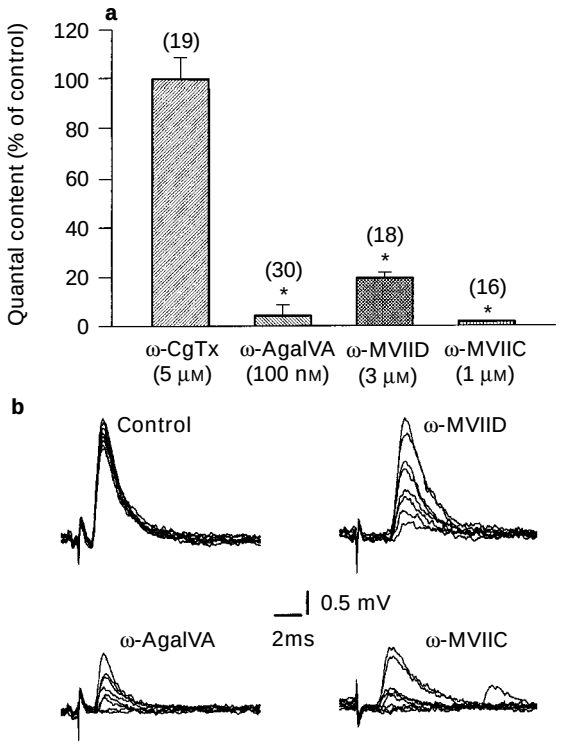

Figure 1 Effect of ω-AgaIVA and ω-conotoxins on nerve-evoked release. (a) The effects of the toxins on quantal content (*m*) as percentage of the control value. In controls, $m = 73 \pm 4.3$ (mean ± s.e.mean, $n = 146$ endplates from 14 muscles). Control fibres were assayed in normal saline solution in the presence of 1.2–1.8 μM (+)-tubocurarine ((+)-Tc). Treated fibres were assayed in the same muscles after 1 h of incubation with the respective toxin. The toxins ω-AgaIVA (100 nM), ω-MVIID (3 μM) and ω-MVIIC (1 μM) strongly reduced the evoked response while ω-CgTx (5 μM) lacked any effect. Each column represents the mean ± s.e.mean of data pooled from 2–4 nerve-muscle preparations (numbers in parentheses are the number of endplates studied). *Student's *t* test (2 tailed), $P < 0.0001$. (b) Representative records of 50 successive e.p.ps elicited at 0.5 Hz. Note that ω-AgaIVA, ω-MVIID and ω-MVIIC reduced the amplitude and increased the variance of the e.p.ps with respect to the control records. In the illustrated records the concentration of (+)-Tc in the normal saline solution was: control, 1.4 μM, ω-AgaIVA, 0.4 μM and ω-MVIID, 0.9 μM. ω-MVIIC was assayed without (+)-Tc. Note the m.e.p.p. at the tail of the record with ω-MVIIC. Stimulation artifacts were reduced for clarity.

Sensitivity of nerve-evoked release to the action of ω-MVIIC and ω-MVIID

Even though, both ω-MVIIC and ω-MVIID were able to reduce strongly transmitter release, this process was much more sensitive to ω-MVIIC ($IC_{50} = 39$ nM) than to ω-MVIID ($IC_{50} = 1.4$ μM) (Figure 2). The sensitivity of transmitter release to the action of ω-MVIIC in the presence of 2 mM Ca^{2+} and 1 mM Mg^{2+} was around one order of magnitude higher than that obtained by Bowersox *et al*. (1995).

Increasing the Ca^{2+} concentration of the bathing medium from 2 to 10 mM in the continuous presence of 3 μM ω-MVIIC induced an increase of ~20 fold in the estimated *m*. E.p.p. amplitudes in the presence of 3 μM ω-MVIIC and 2 mM Ca^{2+} were 0.2 ± 0.03 mV ($n = 10$ endplates from 1 muscle) and increased to 3.4 ± 0.5 mV ($n = 9$ endplates from 1 muscle) when $[Ca^{2+}]_o$ was increased to 10 mM (Figure 2c). Even though the elevation of Ca^{2+} caused a significant increase in transmitter release, the blockade exerted by 3 μM ω-MVIIC in the presence of 10 mM $[Ca^{2+}]_o$ was still very strong. In this experiment, the quantal content of the evoked response in 2 mM $[Ca^{2+}]_o$ was 78.6 ± 8.9 ($n = 9$ endplates from 1 muscle). In this condition, after 1 h of incubating the preparation with 3 μM ω-MVIIC, the quantal content was reduced to 0.2 ± 0.04 ($n = 10$ endplates from 1 muscle). Elevating $[Ca^{2+}]_o$ to 10 mM in the continuous presence of 3 μM ω-MVIIC caused the quantal content to increase to 4.8 ± 1.4 ($n = 9$ endplates from 1 muscle). The increase in release is consistent with the high sensitivity of transmitter release to variations in $[Ca^{2+}]_o$ (Dodge & Rahamimoff, 1967) and with the interference of Ca^{2+} with the binding of ω-conotoxins to their targets (Witcher *et al*., 1993; Kristipati *et al*., 1994). Partial inhibition of transmitter release with either ω-CgTx (Kerr & Yoshikami, 1984) or ω-MVIIC (Bowersox *et al*., 1995) has been shown to be readily reversed by increasing $[Ca^{2+}]_o$.

Sensitivity of nerve- and K^+-evoked release to the action of ω-AgaIVA

In previous work at the mouse neuromuscular junction, it was shown that ω-AgaIVA is able to suppress nerve-evoked release in solutions containing physiological concentrations of Ca^{2+} (2 mM) with an IC_{50} of around 5–20 nM (Protti & Uchitel, 1993; Hong & Chang, 1995; Katz *et al* 1996). It is widely known that the process of transmitter release is highly dependent on $[Ca^{2+}]_o$ and that the relationship between $[Ca^{2+}]_o$ and quantal content becomes steeper at low $[Ca^{2+}]_o$ (Dodge & Rahamimoff, 1967). Therefore, it was of interest to test whether the sensitivity of transmitter release to the action of this toxin could be modified by incubating the preparation in a low Ca^{2+} solution. The concentration-response curve obtained in the presence of 0.9 mM Ca^{2+} and 6 mM Mg^{2+} (Figure 3a) shows that the sensitivity of transmitter release to the action of this toxin ($IC_{50} = 16.8$ nM) was similar to that obtained in normal saline solutions in the diaphragm (Protti & Uchitel, 1993; Hong & Chang, 1995) and the levator auris (Katz *et al*., 1996) preparations of adult mice.

Protti & Uchitel (1993) showed that K^+-evoked release was also strongly reduced by 100 nM ω-AgaIVA. In order to compare the potency with which ω-AgaIVA was able to block nerve and K^+-evoked release, we studied the effects of in-

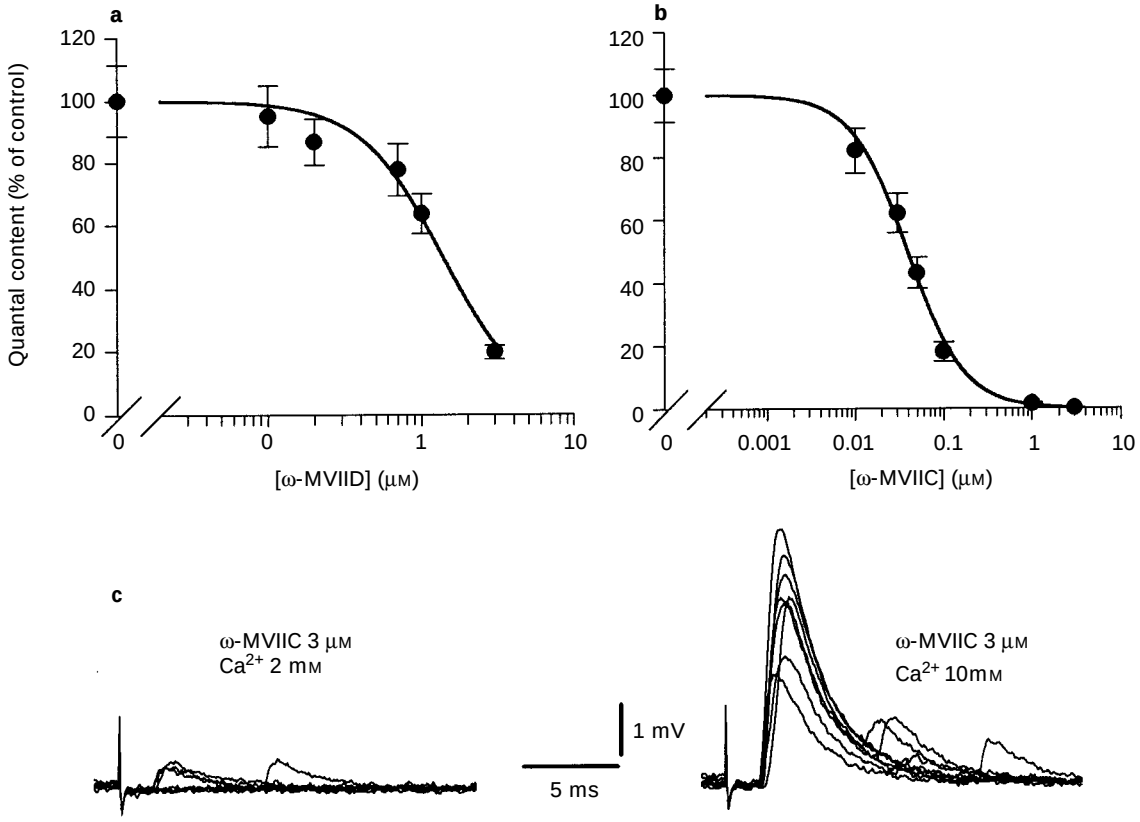

Figure 2 Concentration-dependent blockade of nerve-ending release by ω-MVIID and ω-MVIIC. The concentration response curves show the effects of ω-MVIID (a) and ω-MVIIC (b) on the quantal content of the e.p.p. Each data point represents the quantal content (mean with vertical lines showing s.e.mean) as percentage of the control value of data pooled from 2–4 preparations (at least 10 endplates per muscle). Control fibres were assayed in normal saline solution in the presence of 1.2–1.8 μM (+)-tubocurarine ((+)-Tc). Treated fibres were assayed in the same muscles after 1 h of incubation with the respective toxin. The conotoxin ω-MVIIC blocked transmitter release with an IC_{50} of 39 nM (95% confidence intervals = 34.3 to 44.4 nM; $n_H = 1.4 \pm 0.12$; $r^2 = 0.99$). The conotoxin ω-MVIID blocked transmitter release with an IC_{50} of 1.4 μM (95% confidence intervals = 1 to 1.8 μM; $n_H = 1.6 \pm 0.2$; $r^2 = 0.98$). After almost completely blocking release with 3 μM ω-MVIIC (c), elevating $[Ca^{2+}]_o$ from 2 to 10 mM induced a significant increase ($\sim$20 fold) in the estimated quantal content. Note the dramatic increase in e.p.p. amplitudes after elevation of $[Ca^{2+}]_o$. These are representative records of responses obtained in the same muscle before and after elevation of $[Ca^{2+}]_o$ to 10 mM. Stimulation artifacts were reduced for clarity.

creasing concentrations of this toxin on the K^+-evoked m.e.p.p. frequency (m.e.p.p.). The concentration-response curve obtained in the presence of high K^+ (10 mM) in the external medium shows that the sensitivity of K^+-evoked release to the action of ω-AgaIVA ($IC_{50} = 11.5$ nM) was similar to that found in nerve-evoked release (Figure 3b). In agreement with previous findings (Protti & Uchitel, 1993; Hong & Chang, 1995), spontaneous *f*m.e.p.p. (*f*m.e.p.p. obtained in normal saline solution containing 5 mM K^+), was not affected by either 100 or 300 nM ω-AgaIVA (data not shown).

Reversibility of the effects of ω-AgaIVA on evoked release

The effects of ω-AgaIVA on P-type currents in Purkinje neurones have been shown to be irreversible unless the cells are stepped to very positive voltages while washing out the toxin (Mintz *et al.*, 1992a). At the mouse neuromuscular junction, it has been shown that the effects of ω-AgaIVA on muscle contraction are readily reversible by washing the preparation in normal saline solution without toxin (Bowersox *et al.*, 1995; Hong & Chang, 1995). In contrast, the effects of this toxin on Ca^{2+} presynaptic currents and on nerve-evoked release are not greatly diminished even after 40 min of incubation in normal saline solution without toxin (Protti & Uchitel, 1993). In the above mentioned studies, the reversibility of the effects of ω-AgaIVA on muscle contraction were assayed at body temperature whereas the effects on evoked release and Ca^{2+} presynaptic currents were assayed at room temperature. Therefore, it was of interest to test whether the temperature at which the assays in the presence of ω-AgaIVA are carried out may account for the differences in the reversibility of its effects at the neuromuscular junction. We evaluated the effects of ω-AgaIVA on the e.p.p. quantal content and on the K^+-evoked *f*m.e.p.p. at two different temperatures. The effects of ω-AgaIVA on both types of release were reversed to almost their control values by washing in normal saline solution without toxin (0.6–0.8 ml min^{-1} for 2 h) when the experiment was carried out at 31–33°C (Figure 4). At this temperature, control values and those obtained after washing with normal saline solution without toxin were not significantly different (control *m* versus *m* after toxin washout: $P = 0.2$, $n = 10$ endplates from 2 muscles. Control *f*m.e.p.p. versus *f*m.e.p.p. after toxin washout: $P = 0.7$, $n = 35$ endplates from 2 muscles). By contrast, when the experiments were carried out at 21–22°C the effects of ω-AgaIVA persisted even after 2 h wash (Figure 4). In this case, control values versus those obtained after washing procedure differed significantly (control *m* versus *m* after toxin washout: $P = 0.0004$, $n = 11$ endplates from 2 muscles. Control *f*m.e.p.p. versus *f*m.e.p.p. after wash: $P < 0.0001$, $n = 36$ endplates from 3 muscles).

In order to study whether the ionic environment could modify the reversibility of the effects of ω-AgaIVA at room temperature, we evaluated the effects of toxin washout in the low Ca^{2+}-high Mg^{2+} solutions. In agreement with the results

obtained in normal saline solutions, at 21–22°C, the effects of ω-AgaIVA on evoked release in the low-Ca^{2+}-high Mg^{2+} saline solutions (control, $m = 1.5 \pm 0.14$, $n = 27$ endplates from 2 muscles; treated with 30 nM ω-AgaIVA, $m = 38 \pm 6\%$ of control, $n = 27$ endplates from 2 muscles) could not be reversed even after 2 h of washing out the toxin (after wash, $m = 43 \pm 8\%$ of control, $n = 27$ endplates from 2 muscles).

Effects of ω-AgaIVA and ω-MVIIC on the calcium presynaptic current (I_{Ca})

At the vertebrate neuromuscular junction, by means of the perineurial technique in the presence of K^+ channel blockers (10 mM TEA and 250 μM DAP), it is possible to obtain an outward current, composed of a short (~5 ms) and a long-

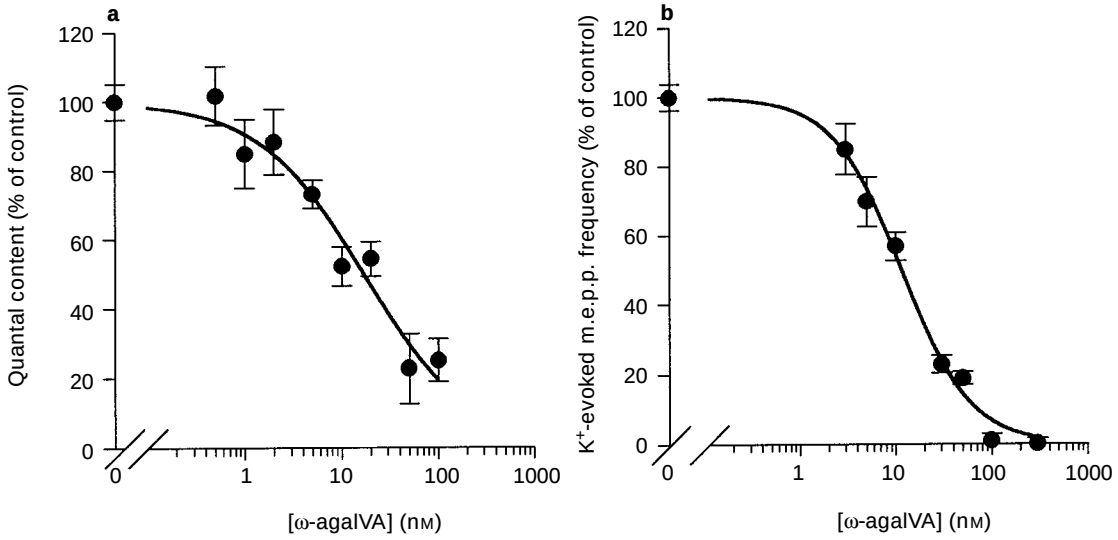

Figure 3 Concentration-dependent blockade of nerve- and K^+-evoked release by ω-AgaIVA. (a) Concentration-response curve showing the effect of ω-AgaIVA in low Ca^{2+}-high Mg^{2+} saline solution. Each data point represents the quantal content (mean ± s.e.mean), expressed as percentage of the control value, of data pooled from 2–3 preparations. Control fibres were assayed in a low Ca^{2+}-high Mg^{2+} saline solution (control $m = 2 \pm 0.1$, mean ± s.e.mean; $n = 130$ endplates from 10 muscles). Treated fibres were assayed in the same muscles after 1 h of incubation with the toxin. ω-AgaIVA blocked transmitter release with an IC_{50} of 16.8 (95% confidence intervals = 11.3 to 24.8 nM; $n_H = 0.81 \pm 0.11$; $r^2 = 0.96$). (b) Concentration-dependent blockade of the K^+-evoked *f*m.e.p.p. (*f*m.e.p.p. in 10 mM K^+ − *f*m.e.p.p. in 5 mM K^+) exerted by ω-AgaIVA. Basal *f*m.e.p.p. was assayed in normal saline solution before incubation of the muscle in a high K^+ medium (10 mM KCl). Treated fibres were assayed in the same muscles after 1 h of incubation in high K^+ saline plus the corresponding toxin concentration. Each data point represents the mean of values obtained in at least 18 fibres; vertical lines show s.e.mean. ω-AgaIVA blocked the K^+-evoked *f*m.e.p.p. with an IC_{50} of 11.5 (95% confidence intervals = 9.6 to 13.7 nM; $n_H = 1.2 \pm 0.1$; $r^2 = 0.99$).

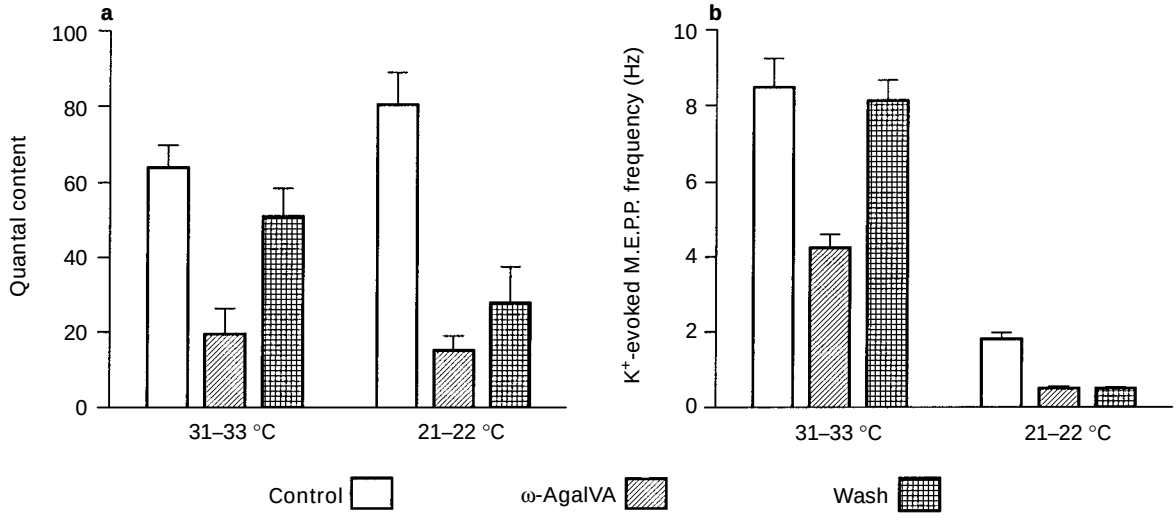

Figure 4 Reversibility of the effects of ω-AgaIVA on transmitter release. (a) The reversibility of the effect of ω-AgaIVA on quantal content (*m*) at two different temperatures. Control fibres were assayed in normal saline solution in the presence of 1.2–1.8 μM (+)-Tc. (b) The reversibility of the effect of ω-AgaIVA on K^+-evoked *f*m.e.p.p. at two different temperatures. Control fibres were assayed in high K^+-saline solution. In (a) and (b), the blockade exerted by 100 nM ω-AgaIVA was evaluated in the same muscles after 1 h of incubation with the toxin. The reversibility of the effect was assayed in the same muscles after washing the preparation with the same solution without toxin for 2 h (~0.6–0.8 ml min^{-1}). Note that at 21–22°C the effects of ω-AgaIVA were not reversed to a great extent by washing whereas at 31–33°C, both types of evoked release were almost restored to their control values by this procedure. Each column represents the mean ± s.e.mean of data pooled from 2–3 nerve-muscle preparations (at least 5 endplates per muscle).

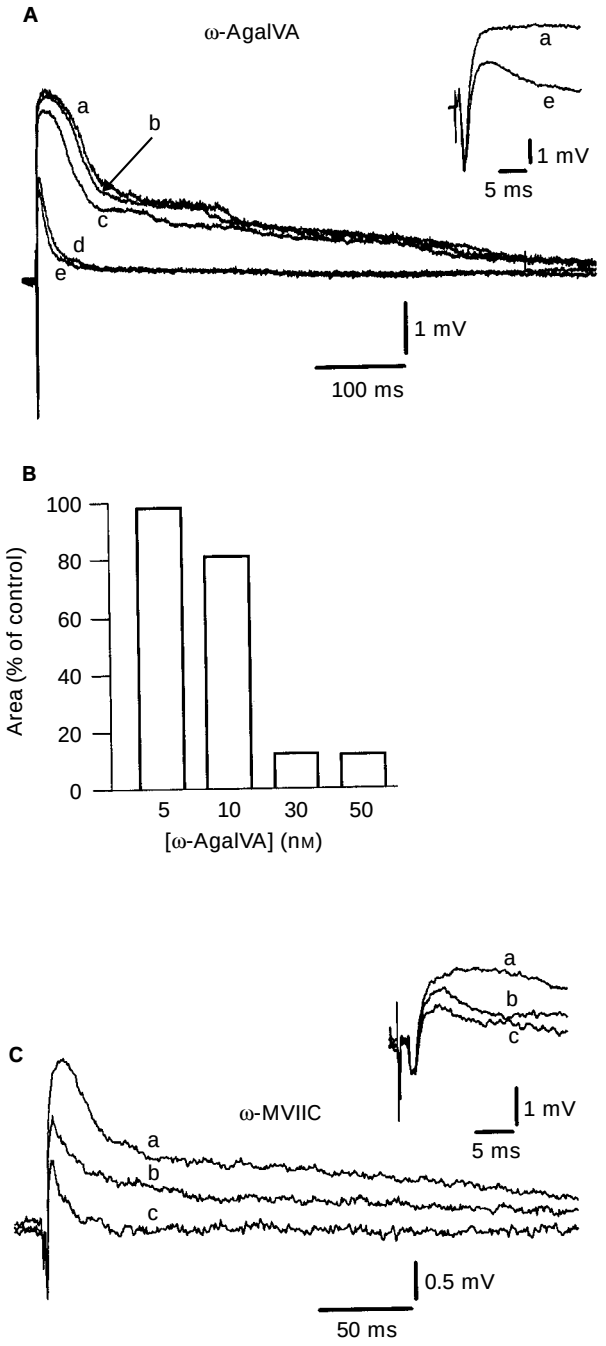

Figure 5 Effect of ω-AgaIVA and ω-MVIIC on the I_{Ca}. (A) Effects of increasing concentrations of ω-AgaIVA on the I_{Ca}. Control I_{Ca} (trace a) was obtained in normal saline solution in the presence of 30 μM (+)-Tc, 250 μM DAP and 10 mM TEA. Traces b, c, d and e were obtained under the same conditions after adding ω-AgaIVA to a final concentration of 5 nM (20 min), 10 nM (25 min), 30 nM (25 min) and 50 nM (15 min), respectively. Times in parentheses indicate the time elapsed in each concentration of ω-AgaIVA before obtaining the corresponding record (the concentration was increased only after two consecutive records without detectable changes in the waveforms had been obtained). The inset shows, on an expanded scale, the control signal (trace a) and the response obtained in the presence of 50 nM ω-AgaIVA (trace e), obtained immediately after records (a) and (e) of the faster sweep. This shows there were no variations in the Na^+ signal throughout the experiment (intermediate records are not shown for clarity). Stimulation frequency was 0.002 Hz. (B) The bar diagram shows the percentage of the control positive area (I_{Ca}) remaining after the maximal effect detected at each ω-AgaIVA concentration in the experiment illustrated in (A). Control positive area = 707 mV ms. (C) Effect of ωMVIIC (1 μM) on the I_{Ca} (trace a) obtained as in (A). Records b and c were taken 40 and 75 min after adding ω-MVIIC to the bath solution, respectively. Records a, b and c of the inset were taken immediately after records a, b and c of the faster sweep and show there were no variations in the Na^+ signal throughout the experiment. Stimulation artifacts were reduced for clarity. Illustrated records represent the results obtained in three (A) or two (C) nerve-muscle preparations.

lasting component (20–500 ms) that reflects the entry of Ca^{2+} into the synaptic terminals upon depolarization (Mallart, 1985a; Penner & Dryer, 1986). These two components were shown to differ in their sensitivity to Ca^{2+} channel blockers and to stimulation frequency (Penner & Dryer, 1986; Mallart *et al.*, 1989; Katz *et al.*, 1995). At the mouse neuromuscular junction, ω-AgaIVA at a concentration of 100 nM was shown to reduce strongly both components of the I_{Ca} (Protti & Uchitel, 1993; Xu & Atchinson, 1996), whereas there is controversy regarding the effects of ω-MVIIC. Lin & Lin-Shiau (1995) showed that ω-MVIIC at a concentration of 1–5 μM blocked only the long-lasting component of the I_{Ca}, while Xu & Atchinson (1996) found that this toxin at 5 μM was able to block both components of the I_{Ca}. As both ω-AgaIVA and ω-MVIIC were very effective in reducing transmitter release, it was of interest to evaluate further their effects on the I_{Ca}.

The effects of applying increasing concentrations of ω-AgaIVA on the I_{Ca} (Figure 5a) showed that very low concentrations of this toxin are able to reduce substantially this current with an IC_{50} that lies between 10 and 30 nM. Considering the effects of 10 nM ω-AgaIVA in two other similar experiments (not illustrated), the I_{Ca} after 20 min of incubation with this concentration of toxin was 40.8 ± 20% ($n = 3$ nerve-muscle preparations) of the control I_{Ca}, as evaluated by comparing the areas under the positive wave before and after the treatment. In these experiments we incubated the preparation for at least 15 min with a given concentration of ω-AgaIVA and increased this concentration only after obtaining two consecutive records without detectable changes in the waveforms. The conotoxin ω-MVIIC was also able to block most of the I_{Ca} (Figure 5c). However, the effects of this toxin had a much slower onset than those of ω-AgaIVA. The slow onset of the effects of ω-MVIIC are consistent with previously obtained results for the blockade of P-type currents in Purkinje neurones (McDonough *et al.*, 1996) and for blocking the I_{Ca} at the mouse neuromuscular junction (Xu & Atchinson, 1996). Figure 5c illustrates the difference between the effects of 1 μM ω-MVIIC at 40 and 75 min after having added it to the bath solution.

Effects of ω-AgaIVA and ω-MVIIC on the calcium activated potassium current ($I_{K(Ca)}$)

Calcium-activated potassium channels sensitive to TEA and charybdotoxin (ChTx) have been shown to activate as a consequence of Ca^{2+} entry to the synaptic terminals upon action potential arrival and to contribute to membrane repolarization in motor synaptic terminals (Mallart, 1985b; Anderson *et al.*, 1988). At the frog neuromuscular junction, calcium-activated potassium channels have been shown to be spatially close and functionally related to the Ca^{2+} channels involved in transmitter release (Robitaille & Charlton, 1992; Robitaille *et al.*, 1993). In frogs, it has been shown that while both FTX and ω-CgTx are able to abolish completely transmitter release, only ω-CgTx is able to block the $I_{K(Ca)}$ (Katz *et al.*, 1995). At the mouse neuromuscular junction, it was recently shown that the $I_{K(Ca)}$ is also involved in regulating transmitter release (Vantapour & Harvey, 1995). Therefore it was of interest to test whether there was a correlation between the concentrations of ω-AgaIVA and ω-MVIIC needed to block release and those needed to block the $I_{K(Ca)}$. Even though a precise quantification of this current is not possible, the records in Figure 6A illustrate that 10 nM ω-AgaIVA were enough to reduce substantially the $I_{K(Ca)}$. This strong inhibitory effect exerted by low concentrations of ω-AgaIVA on the $I_{K(Ca)}$ is in agreement with the

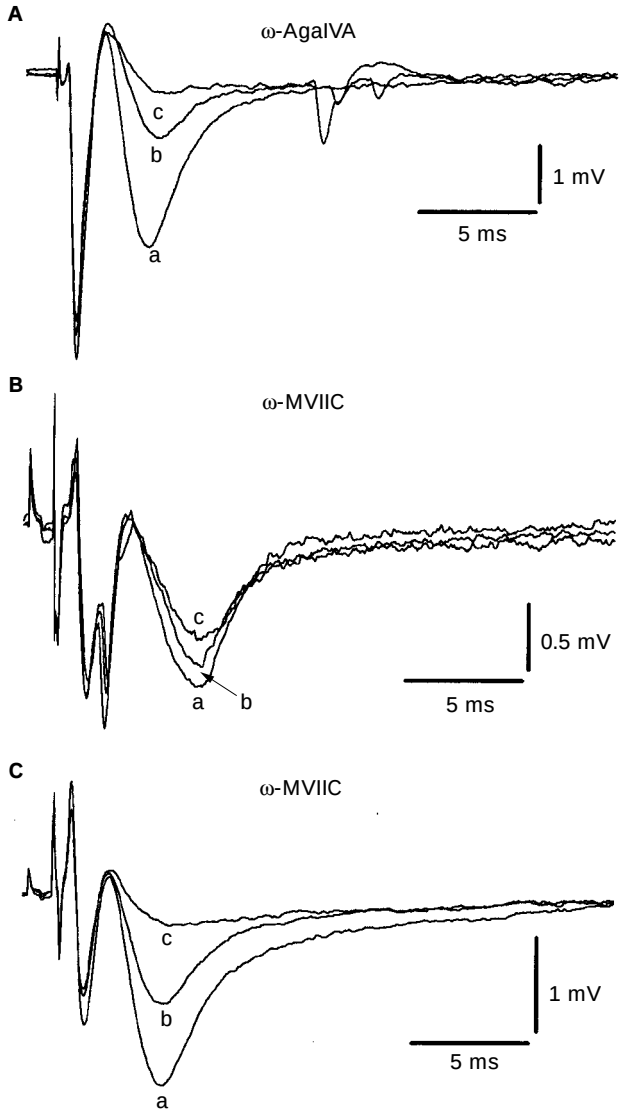

Figure 6 Effect of ω-AgaIVA and ω-MVIIC on the $I_{K(Ca)}$. Control $I_{K(Ca)}$ (traces a in (A), (B) and (C)) were obtained in normal saline solution in the presence of 50 μM (+)-Tc and 1 mM DAP. (A) The strong blockade exerted by ω-AgaIVA on the $I_{K(Ca)}$ after 30 min of adding this toxin to a final concentration of 10 nM (trace b) and 30 nM (trace c). (B) ω-MVIIC only slightly reduced the $I_{K(Ca)}$ after 40 min of adding this toxin to a final concentration of 30 nM (trace b) and 100 nM (trace c). (C) Higher concentrations of ω-MVIIC were able to inhibit strongly the $I_{K(Ca)}$ (trace a). Traces b and c were taken after adding ω-MVIIC to a final concentration of 300 nM (30 min) and 1 μM (90 min), respectively. Each record is the average of 3 successive runs. Stimulation frequency was 0.5 Hz. Stimulation artifacts were reduced for clarity. Illustrated records in (A), (B) and (C) represent the results obtained in two nerve-muscle preparations for each condition.

effects of low concentrations of this toxin on both nerve- and K^+-evoked release and on the I_{Ca}. Due to the slow onset of ω-MVIIC, the effects of low toxin concentrations were difficult to evaluate with accuracy. As shown in Figure 6B, 30 and 100 nM ω-MVIIC for 30 min did not strongly reduce the $I_{K(Ca)}$. Higher concentrations were able to abolish completely this current (Figure 6C) but also after a prolonged incubation. This indicates that the effects of 30 and 100 nM ω-MVIIC could be underestimated. This result is also in agreement with the long incubation times needed to block the I_{Ca} with this toxin. The effects of ω-AgaIVA and ω-MVIIC on the $I_{K(Ca)}$ are in agreement with recently obtained results (Xu & Atchinson, 1996).

Discussion

In the present work we re-evaluated and extended previous findings in relation to the strong inhibitory effects of ω-AgaIVA and ω-MVIIC at the mouse neuromuscular junction (Protti & Uchitel, 1993; Bowersox *et al*., 1995; Hong & Chang, 1995; Wright & Angus, 1995; Katz *et al*., 1996). We also evaluated the effects of ω-MVIID, an ω-conotoxin whose target in rat brain membranes overlaps with that of ω-MVIIC (Monje *et al*., 1993), and found that it strongly reduces transmitter release but with less potency than ω-MVIIC.

Our results show that all the Ca^{2+} dependent processes evaluated in this study (nerve- and K^+-evoked release and the calcium and calcium-activated potassium presynaptic currents) are highly sensitive to low concentrations of ω-AgaIVA (IC_{50} ~10–30 nM). These results are consistent with other data on the effects of ω-AgaIVA at the mammalian neuromuscular junction (Protti & Uchitel, 1993; Hong & Chang, 1995; Bowersox *et al*., 1995; Wessler *et al*, 1995; Protti *et al*., 1996; Katz *et al*., 1996) and with the effects of this toxin on excitatory and inhibitory synaptic transmission in the rat CNS (Turner *et al*., 1992; Takahashi & Momiyama, 1993; Turner & Dunlap, 1995).

Considering the non-linear relationship between external calcium and nerve-evoked transmitter release (Dodge & Rahamimoff, 1967; Augustine & Charlton, 1986), it can be expected that the IC_{50} for the blockade of the current through the calcium channels at the presynaptic membrane will be higher than the IC_{50} for the blockade of transmitter release, as small reductions in calcium entry would cause large reductions in the synaptic response. The IC_{50} obtained for the blockade of P-type currents by ω-AgaIVA in isolated cells is ~1–2 nM (Mintz *et al*., 1992a,b; Wheeler *et al*., 1994a,b; Randall & Tsien, 1995) whereas the IC_{50} for the blockade of Q-type currents is ~90–200 nM (Zhang *et al*., 1993; Wheeler *et al*., 1994a,b; Randall & Tsien, 1995). Therefore, taking into account a power-law relationship between the quantal content of the evoked response and $[Ca^{2+}]_o$ of the type: release $\propto [Ca^{2+}]_o^n$, where n ~4 (Dodge & Rahamimoff, 1967; Augustine & Charlton, 1986), if P-type channels are involved the IC_{50} for the blockade of release should be <1 nM whereas if Q-type channels dominate, the IC_{50} should be ~20 nM (Wheeler *et al*., 1994b). However, the discrimination between P- and Q-type channels based on the evaluation of the IC_{50} for the blockade of transmitter release by ω-AgaIVA may be misleading due to the complexity of the process of release and to the many factors that could lead to error in the estimation of the apparent K_d for the binding of a toxin to the exocytotic channel (Dunlap *et al*., 1995). Unfortunately, a direct evaluation of the calcium currents through the VDCC present at the mammalian motor terminals is not feasible at present. However, the strong inhibitory effects exerted by 10 nM ω-AgaIVA on the perineurial I_{Ca} and the $I_{K(Ca)}$ suggest that the IC_{50} for the blockade of the presynaptic VDCC present at these motor terminals may be close to this value, which is consistent with the effect of ω-AgaIVA on P-type currents (~100% blockade at a concentration of 100 nM; see Dunlap *et al*., 1995).

Consistent with the irreversible nature of the effect of ω-AgaIVA on P-type currents under normal conditions (Mintz *et al*., 1992a), we found that the effects of this toxin on transmitter release could not be reversed by toxin washout at room temperature. However, the blockade can be significantly relieved (>80%) by toxin washout at 31–33°C. A possible explanation for the difference in the reversibility of the effects of this toxin at the two temperatures could be that the rate of toxin unbinding from the channel (K_{off}) is modified not only by voltage, as shown by Mintz *et al*. (1992a) in Purkinje neurones, but also by temperature. As weak chemical bonds are highly sensitive to temperature, the toxin-receptor complex might be more labile at 31–33°C than at room temperature (Hochachka, 1991), which would facilitate the washing out of the toxin at the higher temperature. However, as both high voltage-activated calcium currents (McAllister-Williams & Kelly,

1995) and the process of transmitter release (Hubbard *et al*., 1971) have a high and complex dependence on temperature, further studies should be carried out in order to clarify the effects of temperature on the action of ω-AgaIVA in this system.

With regard to the effects of ω-MVIIC, we found that the sensitivity of transmitter release to the effects of this toxin was higher (IC_{50} $\sim$40 nM) than those previously demonstrated ($IC_{50} \sim 0.3-0.5$ μM) (Bowersox *et al*., 1995). McDonough *et al*. (1996) have shown that the potency for the blockade of both P-type channels in Purkinje neurones and N-type channels in superior cervicle ganglion (SCG) neurones by ω-MVIIC is very sensitive to variations in the ionic composition of the extracellular medium. These authors show that the IC_{50} for the blockade of P-type currents increased from 5 to 50 nM by increasing the Ba^{2+} concentration in the extracellular medium from 2 to 5 mM. This evidence, together with interference of divalent cations with the binding of ω-conotoxins (Witcher *et al*., 1993; Kristipati *et al*., 1994), suggest that the differences found in the IC_{50} for the blockade of transmitter release could be accounted for by the fact that we used a lower concentration of divalent cations in the extracellular medium to study the concentration-response effects of this toxin than those used by Bowersox *et al*. (1995). In addition, the concentration-response curve obtained by Bowersox *et al*. (1995) was performed by incubating the preparation with ω-MVIIC for 30 min whereas in our experiments we used incubation times of more than 1 h.

It was previously shown that ω-MVIIC, at high concentrations (5 μM), could only block the slow component of the I_{Ca} and that it blocked transmitter release only partially if this was elicited in the presence of the voltage-dependent K^+ channel blocker DAP (Lin & Lin-Shiau, 1995). Considering these partial effects, these authors proposed that ω-MVIIC targets only one population of the multiple channels that might be involved in controlling release at the mouse neuromuscular junction. We found that the effect of this toxin, except for the prolonged time necessary to achieve its maximal inhibition, was similar to the strong inhibitory effect exerted by ω-AgaIVA on both outward components (see also Protti & Uchitel, 1993). The long incubation times needed to achieve maximal blockade with ω-MVIIC are consistent with those obtained for the blockade of P-type currents in Purkinje neurones (McDonough *et al*., 1996). The fact that this toxin was able to abolish completely the I_{Ca}, the $I_{K(Ca)}$ and the transmitter release process argue against the hypothesis proposed by Lin & Lin-Shiau (1995). Our results do not exclude the possibility that multiple channel types coexist at the mouse neuromuscular junction and that, under certain conditions, more than one type of channel may be controlling transmitter release (Katz *et al*., 1996) as is known to occur at central synapses (Takahashi & Momiyama, 1993; Mintz *et al*., 1995).

An interesting finding was that the sensitivity of transmitter release to the action of ω-MVIID ($IC_{50} = 1.4$ μM) was much less than that observed upon incubation with ω-MVIIC ($IC_{50} = 39$ nM). This difference was unexpected due to the similar potency with which both synthetic conotoxins are able to displace [^{125}I]-ω-conotoxin MVIIC from the high affinity 'non-N' site on rat brain membranes (Monje *et al*., 1993). This result could be indicating that assay conditions might differentially affect the potency of these two toxins or that the binding of each toxin to this site does not affect the functioning of the channel with equal potency. Alternatively, it may be showing that the VDCC present at these motor terminals may be slightly different from the VDCC targeted by ω-MVIIC and ω-MVIID on rat brain membranes.

In summary, this work shows the strong inhibitory effects exerted by the toxins that target P and Q type VDCC on transmitter release. The almost complete blockade of the I_{Ca} and $I_{K(Ca)}$ presynaptic currents exerted by ω-AgaIVA and ω-MVIIC are consistent with their effects on transmitter release. From these results we can conclude that at the mouse neuromuscular junction Ca^{2+} entry from the extracellular space is accomplished mainly through P (and/or Q) type VDCC which are highly sensitive to both ω-AgaIVA and ω-MVIIC. To discern whether this is a homogeneous or a heterogeneous population of VDCC will need further evaluation with different techniques, in order to assess more directly both the biophysical and pharmacological characteristics of the currents through the channels involved in transmitter release at these synaptic terminals.

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