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Calcium channel subtypes contributing to acetylcholine release from normal, 4-aminopyridine-treated and myasthenic syndrome auto-antibodies-affected neuromuscular junctions

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1 Acetylcholine release at the neuromuscular junction relies on rapid, local and transient calcium increase at presynaptic active zones, triggered by the ion influx through voltage-dependent calcium channels (VDCCs) clustered on the presynaptic membrane. Pharmacological investigation of the role of different VDCC subtypes (L-, N-, P/Q- and R-type) in spontaneous and evoked acetylcholine (ACh) release was carried out in adult mouse neuromuscular junctions (NMJs) under normal and pathological conditions.

2 ω-Agatoxin IVA (500 nM), a specific P/Q-type VDCC blocker, abolished end plate potentials (EPPs) in normal NMJs. However, when neurotransmitter release was potentiated by the presence of the K^+ channel blocker 4-aminopyridine (4-AP), an ω-agatoxin IVA- and ω-conotoxin MVIIC-resistant component was detected. This resistant component was only partially sensitive to 1 μM ω-conotoxin GVIA (N-type VDCC blocker), but insensitive to any other known VDCC blockers. Spontaneous release was dependent only on P/Q-type VDCC in normal NMJs. However, in the presence of 4-AP, it relied on L-type VDCCs too.

3 ACh release from normal NMJs was compared with that of NMJs of mice passively injected with IgGs obtained from patients with Lambert-Eaton myasthenic syndrome (LEMS), a disorder characterized by a compromised neurotransmitter release. Differently from normal NMJs, in LEMS IgGs-treated NMJs an ω-agatoxin IVA-resistant EPP component was detected, which was only partially blocked by calciseptine (1 μM), a specific L-type VDCC blocker.

4 Altogether, these data demonstrate that multiple VDCC subtypes are present at the mouse NMJ and that a resistant component can be identified under 'pharmacological' and/or 'pathological' conditions.

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Abbreviations: 4-AP, 4-aminopyridine; ACh, acetylcholine; EPP, end plate potential; IgG; immunoglobulin; LEMS, Lambert-Eaton myasthenic syndrome; MEPP, miniature end plate potential; NMJ, neuromuscular junction; VDCC, voltage-dependent calcium channel

Introduction

Neurotransmitter release at neuronal synapses is highly dependent on depolarization-induced influx of calcium ions through VDCCs. Different VDCC subtypes have been biophysically and pharmacologically characterized and classified as N-, P/Q-, L-, R- and T-types (Nowycky *et al*., 1985; Tsien *et al*., 1988; Zhang *et al*., 1993). The presence of multiple VDCC subtypes has been highlighted in different synapses throughout the nervous system (Wheeler *et al*., 1994; Wu & Saggau, 1994; Wu *et al*., 1998). At the mammalian NMJ, P/Q-type VDCCs are present (Ousley & Froehner, 1994; Westenbroek *et al*., 1998) and play a major role in evoked ACh release (Uchitel *et al*., 1992; Protti & Uchitel, 1993; Hong & Chang, 1995; Bowersox *et al*., 1995). However, other studies have suggested that different VDCC subtypes may be present at mammalian NMJs (Day *et al*., 1997) and may be involved in both spontaneous (Losavio & Muchnik, 1997) and/or nerve-evoked ACh release (Lin & Lin-Shiau, 1997). L-type VDCCs, for example, can play a role in ACh release at normal NMJs (Atchison, 1989; Urbano & Uchitel, 1999; Correia-de-Sá *et al*., 2000a, b); Urbano *et al*., 2001) and may also contribute to ACh release under pathological conditions; for example, in amyotrophic lateral sclerosis (Fratantoni *et al*., 2000) and in botulinum toxic-poisoned mouse motor nerve terminals (Santafé *et al*., 2000). A role for L-type VDCCs has also been suggested in reinnervating (Katz *et al*., 1996) and newly formed (Sugiura & Ko, 1997; Santafé *et al*., 2001) rat NMJs. An involvement of N-type VDCCs in ACh release at newly formed rat NMJs has also been recently described (Rosato Siri & Uchitel, 1999; Santafé *et al*., 2001). Overall, the question is still open as to how many different VDCC subtypes are present at the

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mammalian nerve terminal and the conditions under which they contribute to transmitter release.

Lambert-Eaton myasthenic syndrome (LEMS) is an autoimmune disorder of neuromuscular transmission characterized by a decreased release of vesicles in response to a single nerve impulse (quantal content), facilitation following high frequency stimulation and a normal post-synaptic response (Lambert & Elmqvist, 1971). LEMS patients have high titres of anti-VDCC auto-antibodies, mainly anti-P/Q-type ($\sim$ 90% of the patients) and anti-N-type VDCC ($\sim$50% of the patients) antibodies. LEMS immunoglobulins (IgGs) have been shown to down-regulate P/Q-type Ca^{2+} currents in different cell types (Magnelli *et al*., 1996; Pinto *et al*., 1998a, b). Passive transfer of LEMS IgGs into mice is sufficient to reproduce the human disease in animals (Lang *et al*., 1983; Kim, 1985). However, little is known about the VDCC subtypes involved in synaptic transmission at the LEMS IgGs-treated NMJ. Changes in the pharmacological sensitivity of VDCCs in mice motor nerve terminals after chronic exposure to LEMS IgGs have been demonstrated (Smith *et al*., 1995; Xu *et al*., 1998).

In the present study we aimed to characterize, relying on elecrophysiological and pharmacological approaches, which VDCC subtypes play the most relevant role in controlling ACh release at normal and pathological NMJs.

Methods

Patients

Sera from six patients with typical clinical and electromyographic features of LEMS were used in this study. The patients' details are briefly summarized in Table 1. All sera, assayed as previously described (Motomura *et al*., 1997), had high titres of anti-VDCC auto-antibodies. All patients had high titres of anti-P/Q-type VDCC antibodies and patient 5 also had anti-N-type VDCC antibodies.

IgGs were prepared by the ethacridine lactate-ammonium sulphate method as previously described (Lang *et al*., 1983). Patients were considered to be positive if their antibodies' titre was three standard deviations above the mean for healthy controls; this value has been determined as >30 pM in the anti-P/Q-type VDCCs assay and >18 pM in the anti-N-type VDCCs assay (Motomura *et al*., 1997). IgGs were also prepared from three healthy controls; one from a healthy 45 year old woman and the other two from pooled plasma of healthy individuals ($n=30$).

LEMS patients were also diagnosed by electromyography. Patients were considered to have LEMS when the compound muscle action potential (CMAP) amplitude, measured from a muscle in the hand, was between 1 and 4 mV and when CMAP amplitude increment during tetanic stimulation was $>100\%$.

Animal work

Male albino (MF1) mice (20–40 g) were used and treated in accordance with British Government Home Office guidelines.

For passive transfer experiments, mice were injected daily intraperitoneally (i.p.) with 1 ml of IgGs (concentration = 10 mg ml^{-1}) for a total period of 9 days. IgGs from healthy controls ($n=3$) and LEMS patients ($n=6$) were coded so that the nature of the IgGs injected into each mouse was unknown until after the data had been analysed. On day 10, mice were sacrificed by raised atmospheric CO_2 followed by cervical dislocation. There was no significant difference in weight in animals receiving different treatments (29.8 ± 0.4 g for LEMS IgGs-treated vs 30.7 ± 0.4 g for control mice) and no obvious sign of muscle weakness was detected in LEMS IgGs-treated mice.

Electrophysiology

Mouse phrenic nerve/hemidiaphragm preparations were excised and pinned out in a 2 ml Sylgard-coated Petri dish containing physiological Krebs solution, continuously bubbled with 95% O_2/5% CO_2 at room temperature (20–23°C). The nerve was stimulated *via* a suction electrode coupled to a pulse generator (GRASS Instruments S48, solid-state square wave stimulator, Quincy, U.S.A.) with an associated stimulus isolation unit. To block muscle contraction, 2.5 μM μ-conotoxin GIIIB (Peptide Institute Inc., Japan) was added to the bath. Nerve-muscle viability was first tested by nerve stimulation in the absence of μ-conotoxin GIIIB. Recordings were made at room temperature (20–23°C). The recording electrodes were connected to an Axoclamp-2A amplifier (Axon Instruments, Foster City, CA, U.S.A.).

Nerve evoked EPPs and MEPPs were recorded intracellularly with conventional glass microelectrodes filled with 3 M KCl (10–15 MΩ resistance; Clark Electromedical Instruments, U.K.) and filtered at 1 kHz. The recording pipette was brought close to the nerve-terminal region under microscopic visualization. End plates were localized by searching for EPPs with fast rise times ($\leqslant$1 ms).

Table 1 Details of the LEMS patients used in this study

LEMS	*Sex*	*Age*	*Cancer*	*Other symptoms*	*Anti-VDCC (P/Q) titre* (pM)	*CMAP* (mV)	*Post-tetanic increment* (%)
1	M	73	Yes (LCLC)	Cerebellar Ataxia	777	2	145
2	F	69	No	–	150	1.8	456
3	M	62	Yes (?)	–	433	0.8	400
4	M	78	Yes (SCLC)	–	500	1	200
5	F	82	No	Dry mouth, constipation	1275*	2.1	614
6	M	27	No	Dry mouth	475	1.5	933

*Patient 5 also had anti-N-type VDCC titre of 1224 pM; all other patients were negative. LCLC = large cell lung carcinoma; SCLC = small cell lung carcinoma.

Protocols

The nerve was stimulated supramaximally with platinum-wire electrodes using standard protocols. After impalement of a muscle fibre, the nerve was first stimulated at 1 Hz for 30 s before recording 30–50 EPPs at this frequency.

Pulses of 0.1 ms duration and of different intensities, depending on the threshold of each preparation, were used for stimulation. The nerve was then left unstimulated for 1 min followed by a train of 50 pulses at 40 Hz. In order to evaluate MEPPs amplitude and frequency, 30–50 traces were recorded and stored for further analysis. Each drug used in the pharmacological studies was directly added to the bath solution and allowed to achieve the final concentration by diffusion. Two different drug application protocols were used. One protocol (referred to as 'acute application' in the relevant results paragraphs and figure legends) was used to study the time course of drugs effects on a single end plate. Alternatively, a second protocol (referred to as 'pre-incubation') consisted in pre-incubating the preparation with the relevant drugs for 1 h and then recording, in the continuous presence of the drug, from many different end plates. Results obtained from this second protocol are expressed as the average of all end plates recorded.

Data analysis

Recordings were rejected if the membrane potential, V_m, was < -60 mV or decreased by more than 5 mV during the recording period or if the 10–90% EPP rise time was >1 ms. The signals were digitized at 12.5 kHz (CED-1401 interface, Science Park Cambridge, U.K.), stored and computer analysed. The software WCP (Whole Cell Program, Strathclyde Electrophysiology Software, John Dempster, 1993–1994) was used for data acquisition and analysis. Each MEPP and EPP was visually inspected before analysis and poor quality traces were discarded.

The mean quantal content (m) was calculated by the direct method (the most accurate method for calculation):

$$m = Avg(Peak_{evoked})/Avg(Peak_{mini}),$$

where Avg is the average, $Peak_{\mathrm{evoked}}$ is the amplitude at the peak of EPP and $\mathrm{Peak}_{\mathrm{mini}}$ is the amplitude at the peak of MEPP.

Averaged EPPs were corrected for non-linear summation (McLachlan & Martin, 1981) using the formula:

$$V_1 = V/[(1 - 0.8\ V)/E],$$

where V_1 is the corrected EPP amplitude, V is the uncorrected EPP amplitude, E is the resting membrane potential and 0.8 is the correction factor. Before correction for non-linear summation, all EPPs and MEPPs were corrected to a standardized membrane potential of -80 mV to correct for changes in driving force due to altered postjunctional membrane potential (Katz & Thesleff, 1957).

Data acquisition, analysis, fitting, averaging and presentation were carried out using a combination of WCP, Excel (Microsoft), SigmaPlot (SPSS), GraphPad Software (Prism and Instat), PowerPoint (Microsoft) and Corel Draw (Corel). Values are expressed as means $\pm$ s.e. Statistical significance (P values in the text and figure legends) was evaluated using the two-tailed Student's t-test. $P<0.05$ was considered to be statistically significant.

Drugs

The bath buffer (Krebs) for the recordings was the following (in mM): NaCl 118, KCl 4.7, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 24.9 and glucose 11. 2.5 mM $CaCl_2$ was added to the Krebs solution the day of the experiment.

$CdCl_2$ was obtained from Acros Organics (UK). ω-Agatoxin IVA, ω-conotoxin GVIA, ω-conotoxin MVIIC were purchased from Peptide Institute, Inc. (Japan) and calciseptine from Alomone Labs (Israel). 4-aminopyridine, nifedipine and S-(−) Bay K 8644 were obtained from Sigma. Nifedipine and S-(−) Bay K 8644 were dissolved in 100% ethanol and stored in the dark at 4°C. Experiments in the presence of nifedipine or S-(−) Bay K 8644 were carried out in the absence of direct illumination to the preparation except for the time required to position the recording electrode. SNX482 was generously donated by Dr G. Dayanithi (Montpellier, France). The toxin was left for an hour at room temperature in the recording solution before use in order to be at neutral pH, where it is more effective.

Results

Effects of Cd^{2+} on ACh release

The effects of Cd^{2+} (500 μM), a non-specific VDCC blocker, were tested on neuromuscular transmission under control conditions. Figure 1A shows that the block caused by this inorganic ion on evoked ACh release was complete (100% inhibition, $n=4$) within 20 min. Cd^{2+} also significantly ($P<0.01$) decreased MEPP frequency (60.4 ± 7.3% reduction; $n=4$) but it did not exert any significant effect on MEPP amplitude (5.2 ± 2.6% inhibition; $n=4$; data not shown), indicating that this inorganic ion has no postsynaptic effect. These results indicate and confirm that both the evoked release and the frequency of spontaneous release are processes dependent on the influx of Ca^{2+} through VDCCs present at the nerve terminal.

Effects of ω-agatoxin IVA on ACh release

As shown in Figure 1B, the application of a relatively high concentration of ω-agatoxin IVA (500 nM), a specific P/Q-type VDCC blocker (Mintz *et al*., 1992), virtually abolished ($94.1\%\pm2.6\%$; $n=8$) EPP amplitude, with a maximal block reached within 25–30 min.

Like Cd^{2+}, also ω-agatoxin IVA (500 nM) significantly reduced MEPP frequency (25.9 ± 9.6% reduction; $n=8$; $P<0.05$), but had no effect on MEPP amplitude (0.1 ± 5.4% inhibition; $n=8$; data not shown).

Effects of other VDCC blockers on ACh release

We further investigated whether L- and N-type VDCCs play any role in the control of ACh release at the adult mouse NMJ. As shown in Figure 1C, ω-conotoxin GVIA (1 μM), an N-type VDCC specific blocker, had no significant effect on EPP amplitude ($n=3$). Nifedipine (5 μM; Figure 1D), a DHP

L-type VDCC antagonist, calciseptine (1 μM; Figure 1E), a potent peptide inhibitor of L-type VDCC and S-(−) Bay K 8644 (1 μM; Figure 1F), an L-type agonist, also had not significant effects.

MEPP frequency and MEPP amplitude were also not significantly inhibited by any of these VDCC blockers.

Effects of VDCC blockers on ACh release using the 'pre-incubation' protocol

Similar results were obtained with the VDCC blockers described above utilizing the 'pre-incubation' protocol (Figure 2). However, some differences between the two protocols were observed and are highlighted here.

Cd^{2+} (500 μM; $n=2$) reduced EPP amplitude from 17.2 ± 1.4 mV to 0.29 ± 0.1 mV (Figure 2C), without affecting MEPP amplitude (from 0.96 ± 0.1 mV to 0.99 ± 0.06 mV; $n=5$; Figure 2A). However, in contrast with the experiments where Cd^{2+} was acutely applied and reduced MEPP frequency, after 1 h of pre-incubation Cd^{2+} effects reversed almost to normal values (0.65 ± 0.1 s^{-1} in Cd^{2+} vs 0.8 ± 0.1 s^{-1} in normal 2.5 mM Ca^{2+} Krebs solution; Figure 2B).

In the 'pre-incubation' experiments, ω-agatoxin IVA inhibited EPP amplitude from a control value of 17.2 ± 1.4 mV to a value of 1.64 ± 0.6 mV ($P<0.01$) and 0.58 ± 0.2 mV ($P<0.01$) at 500 nM ($n=3$) and 1 μM ($n=4$) concentrations, respectively (Figure 2C). The toxin also caused a reduction in MEPP frequency in these 'pre-incubation' experiments from a control value of 0.8 ± 0.1 s^{-1} to values of 0.53 ± 0.1 s^{-1} and 0.44 ± 0.07 s^{-1} at 500 nM and 1 μM toxin ($P<0.05$), respectively (Figure 2B).

The other compounds tested (1 μM ω-conotoxin GVIA, 5 μM nifedipine and 1 μM S-(−) Bay K 8644) were unable to inhibit either evoked or spontaneous ACh release. Only nifedipine significantly increased evoked release from a control value of 17.2 ± 1.4 mV to a value of 24.8 ± 2.2 mV (Figure 2C; $n=3$; $P<0.05$), an effect not seen during the acute applications (Figure 1D).

Finally, we also tested the effect of ω-conotoxin MVIIC (2 μM) which, at this concentration, is known to inhibit both the N- and the P/Q-type VDCCs. ω-Conotoxin MVIIC reduced EPP amplitude from a control value of 22.9 ± 0.7 mV ($n=4$) to a value of 6.9 ± 1.3 mV ($n=4$; $P<0.0001$; data not shown).

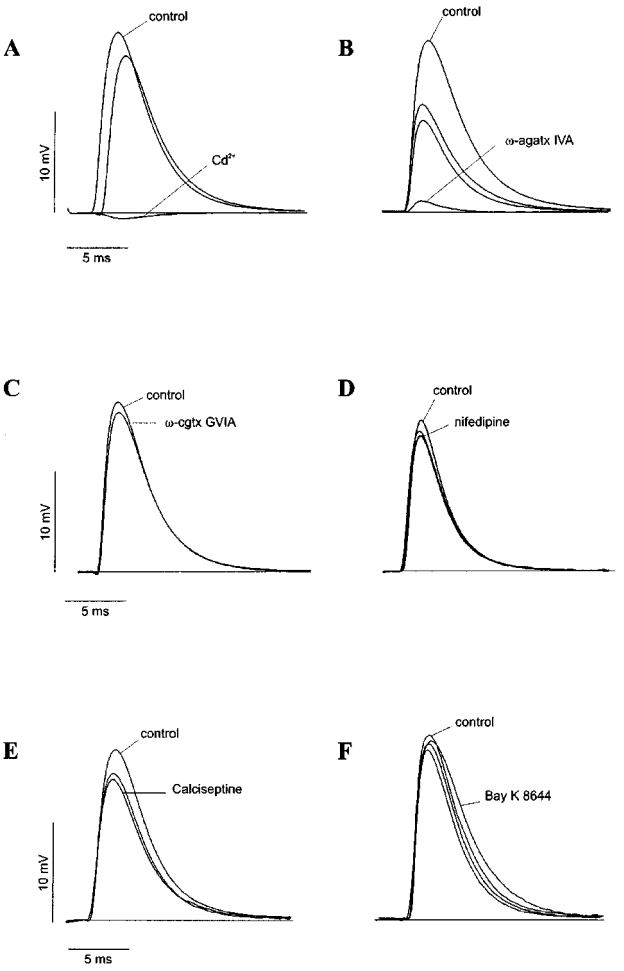

Figure 1 Effect of different VDCCs blockers on EPPs. Representative EPP traces recorded from phrenic nerve/hemidiaphragm preparations in 2.5 mM Ca^{2+} Krebs solution in the absence or presence of Cd^{2+} (500 μM; A), ω-agatoxin IVA (500 nM; B), ω-conotoxin GVIA (1 μM; C), nifedipine (5 μM; D), calciseptine (1 μM; E) or S-(−) Bay K 8644 (1 μM; F). Traces show the effect of the VDCCs blockers 10, 20 and 30 min after their application.

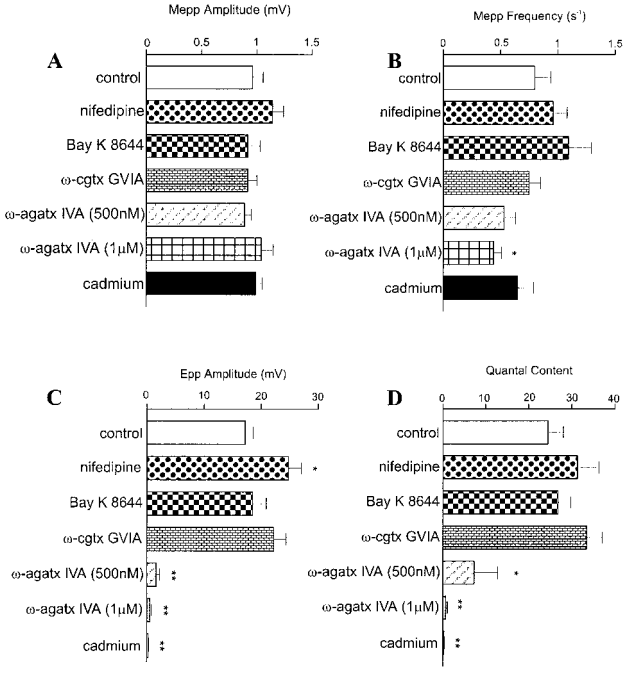

Figure 2 Effect of pre-incubation with specific VDCC blockers on neurotransmitter release at the mouse neuromuscular junction. Bar graphs showing the effect of pre-incubations (1 h; see Methods for description of the protocol) of phrenic nerve/hemidiaphragm preparations with either 2.5 mM Ca^{2+} Krebs solution alone (control; $n=5$) or in the presence of one of the compounds listed below on MEPPs amplitude (A), MEPPs frequency (B), EPPs amplitude (C) and quantal content (D). Results are shown as means+s.e. (*$P<0.05$; **$P<0.01$); n indicates the number of phrenic nerve/hemidiaphragm preparations. Nifedipine (5 μM; $n=3$); S-(−) Bay K8644 (1 μM; $n=3$); ω-conotoxin GVIA (1 μM; $n=3$); ω-agatoxin IVA (500 nM or 1 μM; $n=3$ and $n=4$, respectively) and cadmium (500 μM; $n=3$).

In summary, most of the drugs effects were the same if applied acutely or by using the pre-incubation protocol. We therefore chose to use the pre-incubation protocol for the rest of this study in order to optimize the number of experiments performed while using less of these precious toxins. Only Cd^{2+} and nifedipine showed some differential effects that must be taken in consideration when interpreting the data, as when comparing data from different laboratories (see Discussion).

Effects of 4-AP on ACh release

4-Aminopyridine (4-AP; 300 μM) prevents repolarization of the presynaptic membrane and, as a consequence, drastically increases the amplitude and prolongs the duration of EPPs by blocking K^+ channels located at the nerve terminal (Lundh, 1978; Thomsen & Wilson, 1983). 4-AP increased EPP amplitude and area by $42 \pm 7.4\%$ and of $80 \pm 6\%$ ($n=7$), respectively.

MEPP amplitude was not affected by 4-AP ($0.5 \pm 3\%$ inhibition; $n=3$), thus excluding a direct post-synaptic effect of this compound.

Interestingly, in the continuous presence of 4-AP (300 μM), ω-agatoxin IVA (500 nM; $n=6$) only partially inhibited EPPs area ($45.9 \pm 9.3\%$ inhibition; Figure 3A), confirming that under these conditions of 'pharmacological' enhanced ACh release, an ω-agatoxin IVA-resistant component of release becomes highly relevant (Lin & Lin-Shiau, 1997).

A partial inhibition was also found when ω-agatoxin IVA (500 nM) and ω-conotoxin MVIIC (2 μM) were co-applied in the continuous presence of 4-AP ($50.2 \pm 8\%$ inhibition; $n=2$; Figure 3B), suggesting that, even when all P/Q-type VDCCs are effectively blocked, a component of Ca^{2+} influx sustaining ACh release is still available and belongs to VDCCs other than the P/Q-type.

We studied further this ω-agatoxin IVA- and ω-conotoxin MVIIC-resistant component utilizing the 'pre-incubation' protocol. Phrenic nerve/hemidiaphragm preparations were incubated for 1 h in either 4-AP alone or 4-AP plus one of the above described L-, N- and P/Q-type VDCC specific blockers or a combination of them. SNX 482 (200 nM), a toxin that has been shown to block at least some subtypes of the R-type VDCCs family (Newcomb *et al*., 1998; Wang *et al*., 1999; Tottene *et al*., 2000; Wilson *et al*., 2000), was also tested in these experiments.

In agreement with the above finding in 'acute' experiments, ω-agatoxin IVA inhibited EPP area only by $\sim 50\%$ with respect to the EPPs measured in 4-AP alone (Figure 4A; 147.1 ± 18.3 mV × ms vs 300.1 ± 22.6 mV × ms; $P<0.01$, $n=3$). In the presence of 4-AP, the non-specific VDCC blocker Cd^{2+} completely abolished evoked release (Figure 4A; 5.7 ± 1.1 mV × ms ($n=2$) vs 300.1 ± 22.6 mV × ms ($n=3$); $P<0.01$). This finding crucially demonstrates that also under the extreme release conditions caused by 4-AP, ACh release still relies completely on Ca^{2+} influx *via* VDCCs. Interestingly, in the presence of 4-AP, none of the other specific VDCC blockers was able to significantly reduce nerve-evoked release, when applied singularly.

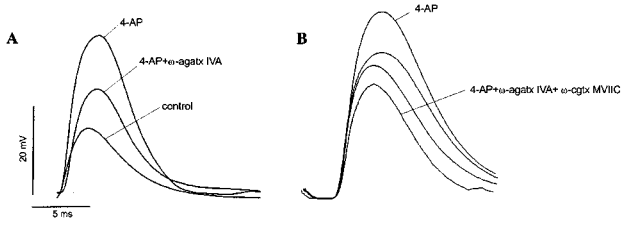

Figure 3 Effect of ω-agatoxin IVA and ω-conotoxin MVIIC in the presence of 4-aminopyridine on EPPs. (A) Representative EPP traces recorded from mouse phrenic nerve/hemidiaphragm preparation in 2.5 mM Ca^{2+} Krebs solution (control), 20 min after the application of 4-aminopyridine (4-AP; 300 μM) and 30 min after the application of ω-agatoxin IVA (500 nM) in the continuous presence of 4-AP (4-AP + ω-agatx IVA). (B) Representative EPP traces recorded from mouse phrenic nerve/hemidiaphragm preparation in 2.5 mM Ca^{2+} Krebs solution and 4-aminopyridine (4-AP; 300 μM) and 10, 20 and 30 min after the application of ω-agatoxin IVA (ω-agatx IVA; 500 nM) and ω-conotoxin MVIIC (ω-cgtx MVIIC; 2 μM) in the continuous presence of 4-AP. Each trace is the average of 20–30 recordings.

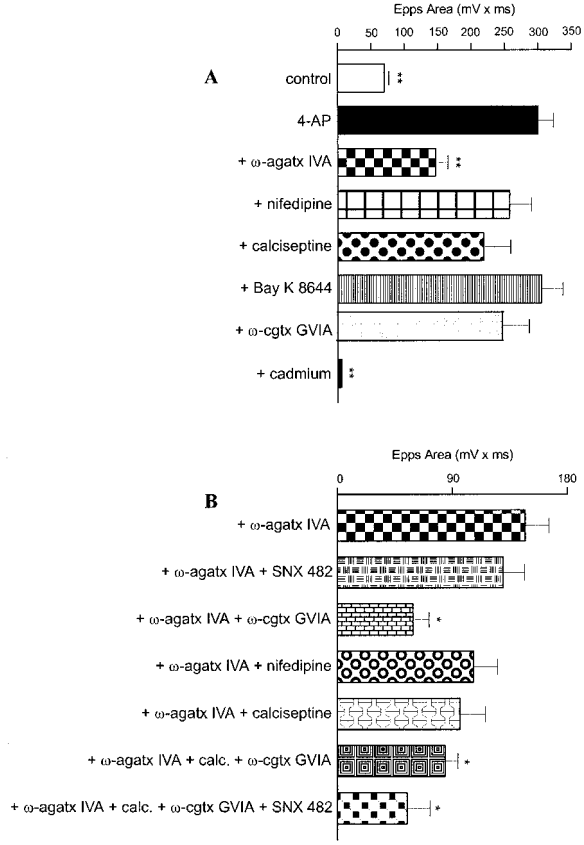

Figure 4 Effect of pre-incubation with specific VDCCs blockers in the presence of 4-aminopyridine on EPPs. Bar graphs showing the effect of pre-incubation of mouse phrenic nerve/hemidiaphragm preparation with 2.5 mM Ca^{2+} Krebs solution alone (control, $n=5$), with 4-aminopyridine (4-AP, 300 μM; $n=3$) or with 4-aminopyridine and one (A) or more (B) of the compounds listed below on EPPs area. Results are shown as means + s.e. (*$P<0.05$; **$P<0.01$); n indicates the number of hemidiaphragm preparations. Nifedipine (5 μM; $n=4$); calciseptine (calc., 1 μM; $n=3$); S-(−) Bay K 8644 (1 μM; $n=3$); ω-conotoxin GVIA (ω-cgtx GVIA; 1 μ M; $n=3$); cadmium (500 μM; $n=2$); ω-agatoxin IVA (ω-agatx IVA, 500 nM; $n=3$); ω-agatoxin IVA and SNX 482 (200 nM; $n=2$); ω-agatoxin IVA and ω-conotoxin GVIA ($n=3$); ω-agatoxin IVA and nifedipine ($n=3$); ω-agatoxin IVA and calciseptine ($n=3$); ω-agatoxin IVA, calciseptine and ω-conotoxin GVIA ($n=5$); ω-agatoxin IVA, calciseptine, ω-conotoxin GVIA and SNX 482 ($n=2$).

However, some synergistic effects were observed. The simultaneous application of ω-agatoxin IVA (500 nM) with ω-conotoxin GVIA (1 μM) caused a further reduction compared with the reduction caused by ω-agatoxin IVA alone (Figure 4B; 59.2 ± 12.8 mV × ms ($n=3$) in ω-agatoxin IVA and ω-conotoxin GVIA vs 147.1 ± 18.3 mV × ms ($n=3$) in ω-agatoxin IVA alone; $P<0.05$). Neither calciseptine alone nor the combination of calciseptine and SNX 482 were able to inhibit the ω-agatoxin IVA- and ω-conotoxin GVIA-resistant components in the presence of 4-AP (Figure 4B; 84.3 ± 10 mV × ms ($n=5$) and 54.7 ± 18 mV × ms ($n=2$) 59.2 ± 12.8 mV × ms ($n=3$)). We also tested the effect of pre-incubation of ω-conotoxin MVIIC (2 μM) in the absence and presence of 4-AP and found that, similarly to ω-agatoxin IVA, this toxin was much less effective in inhibiting the evoked release in the presence of 4-AP than in its absence, as shown in Figure 5. EPPs area was indeed reduced by ω-conotoxin MVIIC, in the absence of 4-AP, by ~76% (133.3 ± 4.1 mV × ms in normal 2.5 mM Ca^{2+} Krebs solution vs 31.4 ± 7 mV × ms in ω-conotoxin MVIIC; $n=4$; $P<0.0001$) and only by ~36% in its presence (372.7 ± 27.8 mV × ms in 4-AP vs 237.7 ± 44.8 mV × ms in 4-AP + ω-conotoxin MVIIC; $n=4$; $P<0.05$).

ACh release at LEMS IgGs-treated NMJs

Table 2 summarizes the pooled data of the electrophysiological findings on NMJs of control IgGs- ($n=20$) and LEMS IgGs-injected ($n=26$) mice.

As shown in previous studies, LEMS IgGs reduced EPP amplitude. Figure 6 shows representative traces of EPPs from mice injected with a control IgG ($\mathbf{A}_1$) or with a LEMS IgG ($\mathbf{A}_2$; LEMS 4; see Table 1 for details). While EPPs recorded from single end plates in control mice appeared to be homogeneous in size, EPPs in LEMS IgGs-treated mice were found to be highly variable.

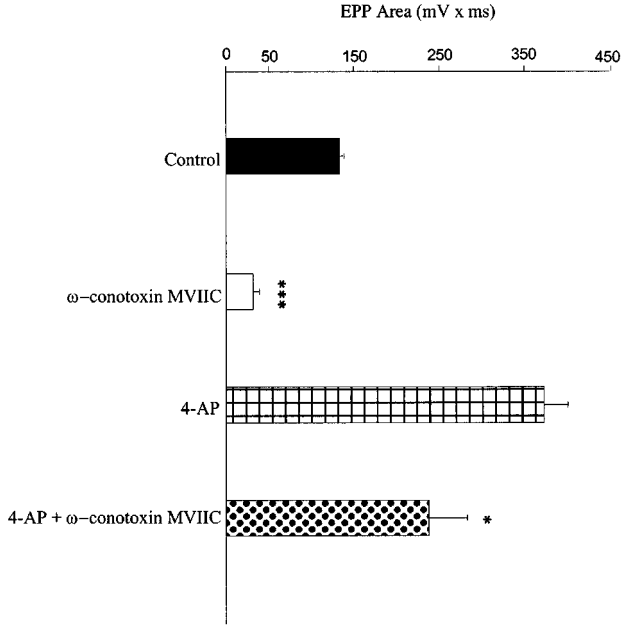

Figure 5 Effect of pre-incubation with ω-conotoxin MVIIC in the absence and in the presence of 4-aminopyridine on EPPs. Bar graph showing the effect of 1 h pre-incubations of mouse phrenic nerve/hemidiaphragm preparations with ω-conotoxin MVIIC (2 μM) in the absence (2.5 mM Ca^{2+} Krebs solution alone; control) or presence of 4-aminopyridine (300 μM; 4-AP) on EPPs area. Results are shown as means + s.e. (*$P<0.05$; ***$P<0.0001$; $n=4$).

Quantal content, calculated with the 'direct method' as the ratio between the averaged EPP amplitude and the averaged MEPP amplitude, was also reduced by injections of LEMS IgGs (Table 2). Moreover, EPP amplitude depression during a train of 50 consecutive stimuli given at high frequency (40 Hz) was significantly less marked in LEMS IgGs-treated preparations than in control preparations (Table 2). The time course of EPP amplitude depression during the train also differed between control and LEMS IgGs-treated preparations (Figure 6B). EPP amplitude decreased gradually during the whole protocol in control NMJs ($t_{1/2}=0.25$ s), whereas it reached its maximum level during the first part of the train in LEMS IgGs-treated NMJs ($t_{1/2}=0.087$ s).

MEPP amplitude was not affected by LEMS IgGs (Table 2). Although electrophysiological recordings showed that neuromuscular transmission was compromised in LEMS IgGs-treated mice, no visible signs of muscle weakness were observed. This may be due to the high safety factor for this species.

Effects of ω-agatoxin IVA on ACh release at the LEMS IgGs-treated NMJ

Figure 7A shows representative EPP traces recorded from LEMS IgGs-treated NMJs (LEMS 4; see Table 1 for details); ω-agatoxin IVA (500 nM) only partially inhibited EPPs amplitude ($48.5 \pm 5\%$; $n=7$; $P<0.001$).

The inhibition was time-dependent with maximal block reached after 30 min from the start of toxin application. The partial block of EPPs caused by ω-agatoxin IVA indicates that in LEMS IgGs-treated NMJs, as in 4-AP-treated NMJs, ω-agatoxin IVA-insensitive Ca^{2+} channels contribute to the evoked ACh release.

ω-Agatoxin IVA had no significant effects on MEPP amplitude (0.9 ± 0.1 mV in control 2.5 mM Ca^{2+} Krebs solution vs 1 ± 0.2 mV in ω-agatoxin IVA; $n=6$) and frequency (0.9 ± 0.03 s^{-1} in control 2.5 mM Ca^{2+} Krebs solution vs 0.8 ± 0.16 s^{-1} in ω-agatoxin IVA; $n=6$).

The same reduction in the blocking activity of ω-agatoxin IVA was observed in NMJs of mice treated with IgGs from two other LEMS patients (LEMS 5 and 6; see Table 1 for details). In these two cases, the blocking effects of ω-agatoxin IVA were not tested 'acutely' as for the first case described above, but with the 'pre-incubation' protocol. Interestingly, NMJs treated with LEMS 6 IgG showed a more marked reduction in EPP amplitude (35%; $P<0.01$; $n=6$) and these EPPs (Figure $7B_1$) were only slightly blocked (~28% inhibition) by ω-agatoxin IVA (9.9 ± 1.2 mV in ω-agatoxin IVA ($n=3$) vs 13.8 ± 1.1 mV in control ($n=6$)). On the other hand, NMJs treated with LEMS 5 IgG showed only a 23% reduction of EPP amplitude ($P<0.05$; $n=7$) and these EPPs (Figure $7B_1$) were significantly blocked (~71.3% inhibition) by agatoxin IVA (4.7 ± 1 mV in ω-agatoxin IVA ($n=3$) vs 16.4 ± 1.2 mV in control ($n=7$); $P<0.01$).

It is worth noting that LEMS 6 IgG was also more effective in inducing the physiological changes of the passive transfer paradigm. In NMJs from mice treated with control IgGs, EPPs amplitude (Figure $7B_1$) and quantal content

(Figure 7B$_2$) were almost completely abolished (~93% inhibition) by ω-agatoxin IVA (1.5±0.6 mV in ω-agatoxin IVA ($n=7$) vs 21.2±1.9 mV in control ($n=3$); $P<0.01$), as in non-injected mice.

Effects of other VDCC blockers on ACh release at the LEMS IgGs-treated NMJ

The sensitivity of LEMS IgGs-treated NMJs to other VDCC blockers was then examined (Figure 8). Nifedipine (5 μM; Figure 8A) and calciseptine (1 μM; Figure 8B), two L-type VDCC specific blockers, caused no statistically significant inhibition of EPP amplitude (17.6±5.3% ($n=3$) and 27.5±13% ($n=4$), respectively). The amplitude and the frequency of the MEPPs were not significantly affected by these drugs (data not shown). However, the simultaneous application of calciseptine (1 μM) and ω-agatoxin IVA (500 nM) to a LEMS IgGs-treated NMJ caused a larger reduction in EPP amplitude (64±0.8% ($n=5$; $P<0.001$; Figure 8C) compared to ω-agatoxin IVA alone and this effect reached statistical significance (Figures 7A and 8B). The amplitude and the frequency of MEPPs were not significantly affected even by the simultaneous application of these drugs (amplitude: 0.97±0.1 mV to 0.95±0.2 mV ($n=2$); frequency: 0.79±0.1 s^{-1} to 0.7±0.2 s^{-1} ($n=4$)).

Discussion

In this study, we have investigated the presence of different VDCC subtypes at the adult mouse NMJ and their role in the control of spontaneous and nerve-evoked ACh release. This work is a comprehensive pharmacological study of a wide range of VDCC blockers effect on either normal or pathological phrenic nerve/hemidiaphragm preparations.

In agreement with previous findings in mammalian NMJs (Protti & Uchitel, 1993; Hong & Chang, 1995; Protti *et al*., 1996), we have found P/Q-type VDCC to be the predominant Ca^{2+} channel subtype coupled to the evoked release process under control conditions. In contrast, neither N- nor L-type specific VDCC blockers had any significant effect in reducing nerve-evoked ACh release. Under these conditions, however, the 'pre-incubation' of the preparation with nifedipine caused a small but significant increase in EPP amplitude compared to control recordings. This surprising result of potentiation rather than inhibition of transmitter release after blocking L-type VDCCs has been previously reported by Sugiura & Ko (1997) in immature mammalian and amphibian NMJs. These authors hypothesized that Ca^{2+} entry through L-type VDCCs may trigger the release of a neuromodulator which, by binding to a receptor coupled to a PTX-sensitive G-protein, could mediate the inhibition of ACh release. The blockade of L-type VDCC with nifedipine would prevent this G-protein-mediated inhibition and result in an increased transmitter release. Nifedipine could also cause the blockade of K^+ channels present at the nerve terminal (like 4-AP) and this in turn would cause depolarization and ACh release. Indeed, nifedipine has previously been reported to block cloned K^+ channels (Grissmer *et al*., 1994) as well as K^+ channels expressed in the heart (Zhang *et al*., 1997).

The role of VDCC subtypes in spontaneous release was also investigated. It is known that both nerve-evoked release and MEPP frequency are, in different ways, dependent on

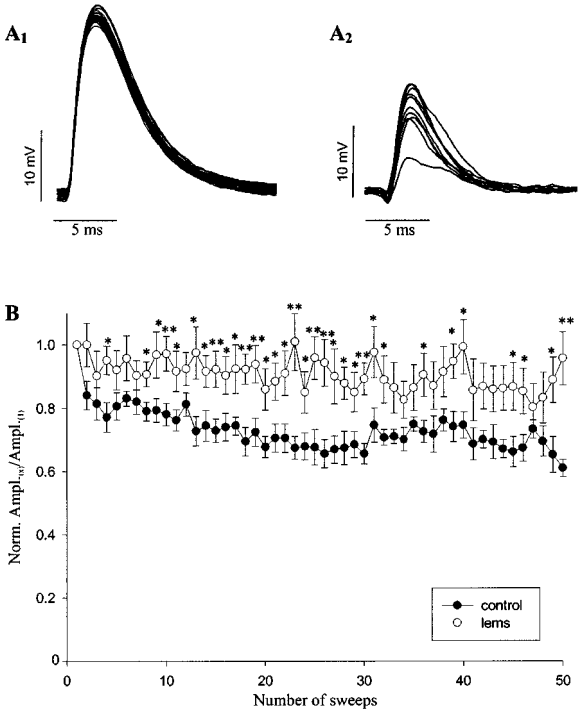

Figure 6 Effect of LEMS IgGs on neurotransmitter release at the mouse neuromuscular junction. Representative EPP traces recorded from control IgGs-treated (A_1) and LEMS IgGs-treated (A_2, LEMS 4; see Table 1) mouse phrenic nerve/hemidiaphragm preparations in 2.5 mM Ca^{2+} Krebs solution. Note that, in each experiment, traces are recordings from a single end plate. (B) Graph plotting the normalized mean EPP amplitude against a train of 50 sweeps delivered at 40 Hz. Before averaging across experiments, EPPs amplitude of each recording ($Ampl._{(X)}$) was normalized by that obtained in the first recording ($Ampl._{(1)}$). Recordings were obtained from phrenic nerve/hemidiaphragm preparations of mice immunized with either control IgGs ($n=20$) or LEMS IgGs ($n=26$). Error bars represent s.e.; n indicates the number of experiments. $^*P<0.05$; $^{**}P<0.01$.

Table 2 Electrophysiological properties of NMJs from mice immunized with control or LEMS IgGs

	EPPs amplitude (mV) (% reduction)	*Quantal content* (% reduction)	*% inhibition at 40 Hz*	*MEPPs amplitude* (mV)	*MEPPs frequency* (s^{-1})
Control ($n=20$)	19.7±1.8	27.8±2.7	33.3±1.74	0.97±0.09	1.04±0.2
LEMS ($n=26$)	13.9±1.1	20.6±2.3	22±1.1	0.86±0.07	0.83±0.17
	$^{**}P<0.01$	$^*P<0.05$	$^{***}P<0.001$		
	(29.5%)	(25.9%)			

All results are expressed as means±s.e.; n indicates the number of hemidiaphragm preparations. $^*P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$.

intracellular Ca^{2+} concentration (Matthews & Wickelgren, 1977).

Our results, obtained under physiological conditions, of ω-agatoxin IVA causing a significant reduction of MEPP frequency, strongly suggest that the P/Q-type VDCC is the major Ca^{2+} channel subtype involved also in spontaneous release. This result is in contrast with the findings of Losavio & Muchnik (1997), who showed a role for L- and N-type, but not P/Q-type VDCCs in the regulation of spontaneous release. The reasons for this discrepancy are unclear but might include the use of different animals (mice vs rats) and of slightly different concentrations of VDCC blockers (Protti *et al*., 1991).

The inorganic ion Cd^{2+}, a non-specific VDCC blocker which completely abolished nerve-evoked release, significantly reduced MEPP frequency 20 min after its application, but the effect apparently reversed over time and no significant changes in MEPP frequency could be recorded 1 h after its application. This finding is in line with previous reports on the effects of Cd^{2+} on spontaneous release at mammalian NMJs. MEPP frequency has been shown either to be not significantly affected (Forshaw, 1977; Porter & Wray, 1996), or to be increased (Nishimura *et al*., 1984; Braga & Rowan, 1994) by Cd^{2+} ions. Part of the mechanism by which Cd^{2+} influences spontaneous ACh release is thought to be intracellular, possibly interfering with intracellular Ca^{2+} stores (Nishimura *et al*., 1984). The differential effects of Cd^{2+} on spontaneous release in these published papers may be explained by the different protocols used. During acute exposure, Cd^{2+} mainly binds to the pore of the channel hence preventing the passage of Ca^{2+} ions and ACh release (Nachshen, 1984). In contrast, after pre-incubation, Cd^{2+} might also permeate the cells and cause ACh release either directly or by releasing intracellular Ca^{2+} from stores.

The above results confirm previous findings suggesting that only Cd^{2+}- and ω-agatoxin IVA-sensitive (P/Q-type) Ca^{2+} channels contribute to both spontaneous and nerve-evoked ACh release at the mouse NMJ (Protti & Uchitel, 1993; Hong & Chang, 1995; Protti *et al*., 1996). However, electrophysiological studies have demonstrated that multiple types of VDCCs exist in neuron somata (Dunlap *et al*., 1995) as well as in central synapses (Takahashi & Momiyama, 1993; Wheeler *et al*., 1994). In particular, the presence of multiple VDCC subtypes has been demonstrated in mature mouse (Carlin *et al*., 2000) and rat (Magnelli *et al*., 1998) spinal motoneurons. Furthermore, Penner & Dreyer (1986) have previously suggested the presence of at least two distinct subtypes of Ca^{2+} channels in mouse motor nerve terminals.

We therefore conducted further experiments in order to evaluate if different VDCC subtypes contribute to ACh release from mouse motor nerve terminals under 'modified' and autoimmune (LEMS auto-antibodies) conditions. Indeed, in LEMS, patients producing VDCC auto-antibodies release less ACh from their nerve terminals; furthermore, this condition is treated with 4-AP, which potentiates ACh release. We indeed found, in both these physiological conditions, an ω-agatoxin IVA- and ω-conotoxin MVIIC-resistant Ca^{2+} channel component contributing to ACh release from mouse motor nerve terminals.

Although we could not confirm the results by Lin & Lin-Shiau (1997), showing a synergistic effect of ω-agatoxin IVA

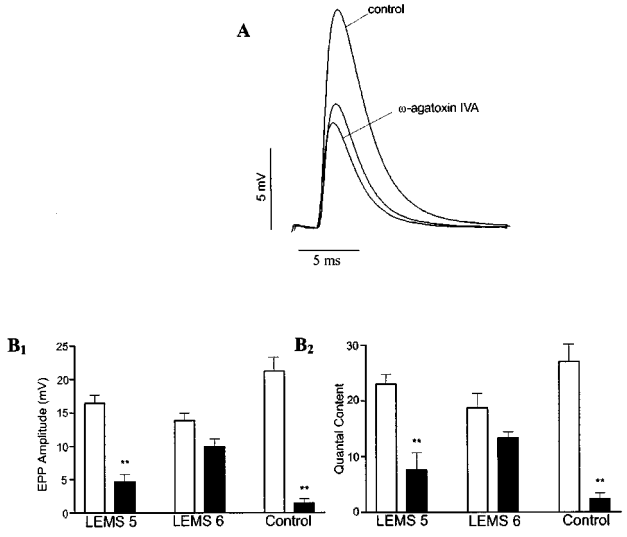

Figure 7 Effect of ω-agatoxin IVA on EPPs of LEMS IgGs-treated mouse neuromuscular junctions. (A) Representative EPP traces recorded from a LEMS-treated mouse phrenic nerve/hemidiaphragm preparation (LEMS 4; see Table 1) in 2.5 mM Ca^{2+} Krebs solution (control), 15 and 30 min after the application of ω-agatoxin IVA (500 nM). Each trace is the average of 20–30 traces. (B_1 and B_2) Bar graphs showing the effect of pre-incubations (1 h; see Methods for description of the protocol) of mouse phrenic nerve/hemidiaphragm preparations treated with LEMS IgGs (LEMS 5 and LEMS 6; see Table 1) or with healthy control IgGs (control). Recordings of EPPs amplitude (B_1) and quantal content (B_2) were carried out in the presence of 2.5 mM Ca^{2+} Krebs solution alone (empty bars; $n=7$) or with ω-agatoxin IVA (500 nM, black bars, $n=3$). Results are shown as means+s.e. ($^{**}P<0.01$); n indicates the number of hemidiaphragm preparations.

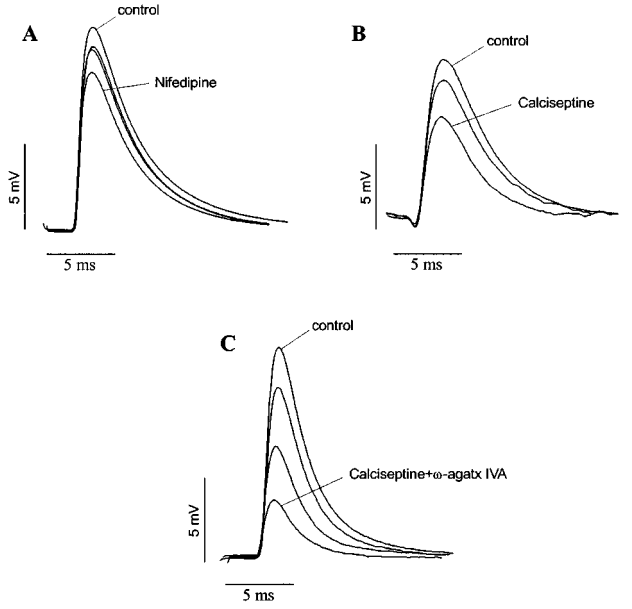

Figure 8 Effect of nifedipine, calciseptine and ω-agatoxin IVA on EPPs of LEMS IgGs-treated mouse neuromuscular junctions. Representative EPP traces recorded from mouse phrenic nerve/hemidiaphragm preparation (LEMS 5; see Table 1 for details) in 2.5 mM Ca^{2+} Krebs solution (control) and 10, 20 and 30 min after application on (A) nifedipine (5 μM) or (B) calciseptine (1 μM) or (C) calciseptine (1 μM) and ω-agatoxin IVA (500 nM). Each trace is the average of 20–30 traces.

and ω-conotoxin MVIIC, we did find a synergistic effect of ω-agatoxin IVA and ω-conotoxin GVIA in their ability to depress EPPs in the presence of 4-AP.

These results support the hypothesis that VDCCs other than the P/Q-type become involved in ACh release in the presence of 4-AP; in particular, our results suggest that both N- and 'R'-type VDCCs become important under this pharmacological condition.

N-type VDCCs presence in normal mammalian nerve terminals has already been previously reported and these channels are identified by their sensitivity in ω-conotoxin GVIA (Protti *et al*., 1991; Rossoni *et al*., 1994; Santafé *et al*., 2000). The presence of Ca^{2+} channels resistant to all the known VDCC specific blockers ('R'-type) contributing to evoked ACh release, as shown here and by Lin and Lin-Shiau, is less established and characterized. Interestingly, these 'R'-type VDCCs appear to be insensitive to SNX 482 under our experimental conditions. SNX 482 has been previously reported to selectively block some but not all R-type VDCCs (Newcomb *et al*., 1998; Wang *et al*., 1999; Tottene *et al*., 2000) present in several different systems. We are not aware of other reports where this toxin has been tested on NMJs.

In the LEMS IgGs-treated mice, EPP amplitude was significantly reduced compared to that in control NMJs and ω-agatoxin IVA was much less effective in inhibiting EPP amplitude in these LEMS IgGs-treated NMJs. Interestingly, the extent of EPP inhibition caused by LEMS IgGs seems to be inversely correlated to the extent of EPP inhibition caused by ω-agatoxin IVA in the same junctions. The simultaneous application of ω-agatoxin IVA and calciseptine to the LEMS IgGs-treated NMJs caused greater EPP inhibition than that caused by the individual toxins, indicating that L-type VDCCs do play a role in the control of nerve-evoked release under this pathological condition.

The 'recruitment' of a DHP-sensitive L-type VDCC component in LEMS IgGs-treated mice NMJs as well as in LEMS IgGs-treated mice motoneurons has been previously reported (Smith *et al*., 1995; Garcia & Beam, 1996; Xu *et al*., 1998). However, DHP drugs are known to be non-selective Ca^{2+} channel blockers when used at micromolar concentrations (Diochot *et al*., 1995). For this reason, we tested the possible involvement of L-type VDCCs in ACh release using calciseptine, a more selective L-type VDCC blocker (de Weille *et al*., 1991).

Our results with calciseptine confirm the hypothesis that L-type VDCCs become involved in the control of synaptic transmission at LEMS IgGs-treated mouse NMJ. L-type VDCCs in mammalian motor nerve terminals (Atchison & O'Leary, 1987; Atchison, 1989; Katz *et al*., 1996; Sugiura & Ko, 1997; Urbano & Uchitel, 1999; Correia-de-Sá *et al*., 2000a, b; Santafé *et al*., 2000) have been previously identified; moreover, a role of these channels in the control of ACh release during development (Gray *et al*., 1992; Fu & Huang, 1994) and pathological conditions (Fratantoni *et al*., 2000) has been demonstrated. Our results show for the first time that, in LEMS IgGs-treated NMJs, both L- and 'R'-type VDCCs are 'recruited' for a functional role when a deficit in P/Q-type VDCCs is occurring, like in LEMS.

Compensatory increases in specific components of Ca^{2+} currents have already been described. An increase in the proportion of a residual 'R'-type Ca^{2+} current and a correspondent decrease in the proportion of P-type current after 15–22 h of incubation of cerebellar Purkinje neurons in the presence of LEMS IgGs has been reported by Pinto *et al*. (1998a). Gillard *et al*. (1997) found similar compensatory mechanisms using anti-P/Q-type antisense oligonucleotides in the same type of cells.

The physiological significance of the expression of multiple VDCC subtypes is still not completely clear. Ca^{2+} channel subtypes differ from each other for their distinct biophysical properties, differential modulation and variable coupling to intracellular processes (Neveu *et al*., 1994; Arnot *et al*., 2000).

The existence of different Ca^{2+} channel subtypes at the nerve terminal would allow a more flexible and versatile regulation of ACh release, especially under non-physiological conditions. Different VDCC subtypes may play important and selective roles during development and regeneration of NMJs and in pathological conditions when transmitter release is compromised.

Finally, the presence of multiple VDCCs at the NMJ is likely to represent the basis for the clinical efficiency of compounds such as 4-AP, that rely on the 'recruitment' of 'spared' VDCCs in order to potentiate release from compromised NMJs. A direct potentiation of selective VDCC subtypes to treat secretory deficits is another approach worth considering.

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