Calcium channels involved in synaptic transmission at the mature and regenerating mouse neuromuscular junction

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1. The involvement of the different types of voltage-dependent calcium channels (VDCCs) in synaptic transmission at the mature and newly formed mammalian neuromuscular junction was studied by evaluating the effects of L-, P/Q- and N-type VDCC antagonists on transmitter release in normal and reinnervating levator auris preparations of adult mice.

2. Nerve-evoked transmitter release was blocked by ω-agatoxin IVA (ω-AgaIVA), a P/Q-type VDCC blocker, both in normal and reinnervating endplates (100 nM ω-AgaIVA caused > 90% inhibition). The N-type VDCC antagonist ω-conotoxin GVIA (ω-CgTX; 1 and 5 μM), as occurs in normal preparations, did not significantly affect this type of release during reinnervation. Nitrendipine (1–10 μM), an L-type VDCC blocker, strongly antagonized release in reinnervating muscles (~40–60% blockade) and lacked any effect in normal preparations.

3. In reinnervating muscles, spontaneous release was not dependent on Ca^{2+} entry through either P- or L-type VDCCs. Neither 100 nM ω-AgaIVA nor 10 μM nitrendipine affected the miniature endplate potential (MEPP) frequency or amplitude.

4. At the newly formed endplates, K^+-evoked release was dependent on Ca^{2+} entry through VDCCs of the P-type family (100 nM ω-AgaIVA reduced ~70% of the K^+-evoked MEPP frequency). L-type VDCCs were found not to participate in this type of release (10 μM nitrendipine lacked any effect).

5. In reinnervating muscles, the L-type VDCC blocker, nitrendipine (10 μM), provoked a significant increase (~25%) in the latency of the evoked endplate potential (EPP). This drug also caused an increase (~0·3 ms) in the latency of the presynaptic currents. The P/Q- and N-type VDCC blockers did not affect the latency of the EPP.

6. These results show that at newly formed mouse neuromuscular junctions, as occurs in mature preparations, VDCCs of the P-type family play a prominent role in evoked transmitter release whereas N-type channels are not involved in this process. In addition, signal conduction and transmitter release become highly sensitive to nitrendipine during reinnervation. This suggests that L-type VDCCs may play a role in synaptic transmission at the immature mammalian neuromuscular junction.

Ca^{2+} is a key link between the arrival of an action potential at the synaptic terminal and the release of neurotransmitter (Katz, 1969). The rapid increase in $[Ca^{2+}]_i$ that occurs upon depolarization of the terminal membrane is accomplished by the opening of voltage-dependent calcium channels (VDCCs) that allow the entry of Ca^{2+} from the extracellular space (Llinás, Steinberg & Walton, 1976). Several types of VDCC have been described based on their biophysical and pharmacological characteristics and they have been classified into the types T, L, N and P (Nowicky, Fox & Tsien, 1985; Llinás, Sugimori, Hillman & Cherksey, 1992). More recently, the Q-type, a channel closely related to the P-type, and the R-type, a channel resistant to any of the drugs and toxins commonly used to block and discriminate the other types of VDCC, have been described (Zhang, *et al.* 1993). Many of these VDCCs, particularly the N-, L- and P/Q-types, have been shown to play a role in Ca^{2+} entry during synaptic transmission at different synapses (for review see Olivera, Miljanich, Ramachandran & Adams, 1994).

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At the mature mouse neuromuscular junction, synaptic transmission is mediated by Ca^{2+} entry through channels of the P-type family (Uchitel, Protti, Sánchez, Cherksey, Sugimori & Llinás, 1992; Protti & Uchitel, 1993; Hong & Chang, 1995; Bowersox *et al.* 1995). In this preparation, ω-agatoxin IVA (ω-AgaIVA) a P- and Q-type VDCC blocker (Mintz, Venema, Swiderek, Lee, Bean & Adams, 1992; Zhang *et al.* 1993; Olivera *et al.* 1994) was shown to exert a strong inhibitory effect on [^{3}H]ACh release (Wessler, Dooley & Lohr, 1995), on calcium presynaptic currents (Protti & Uchitel, 1993) and on both neurally and K^+-evoked transmission (Protti & Uchitel, 1993; Hong & Chang, 1995). The complete blockade of evoked transmitter release and presynaptic Ca^{2+} currents exerted by low concentrations of ω-AgaIVA, together with the lack of effects of the L- (Penner & Dryer, 1986; Atchinson, 1989) and N-type (Protti, Szczupak, Scornik & Uchitel, 1991; Bowersox *et al.* 1995) VDCC blockers, dihydropyridines and ω-conotoxin GVIA (ω-CgTX), respectively, suggest that, under normal conditions, only VDCCs of the P-type family (P/Q) are involved in evoked transmitter release in this system. However, there is evidence that indicates that both L- and N-type channels are also present in mouse motor terminals (Atchinson, 1989; Protti *et al.* 1991).

At the immature vertebrate neuromuscular junction EPPs have a lower amplitude, a lower quantal content and a longer latency than EPPs in normal muscles (Bennett, McLachlan & Taylor, 1973; Dennis & Miledi, 1974*a*, *b*; Tonge, 1974). Changes in the density and distribution of ionic channels as well as in action potential propagation and the safety factor for conduction along immature axons and terminal membranes have been described (Bostock, Sears & Sherrat, 1981; Angaut-Petit & Mallart, 1985*a*, *b*). Also, during the course of maturation of the vertebrate neuromuscular junction, changes in the pharmacology of the VDCC coupled to the release process were reported in avian (Gray, Brusés & Pilar, 1992) and amphibian (Fu & Huang, 1994) preparations. Little is known, however, about the pharmacology of the Ca^{2+} channels coupled to the release process in recently formed synapses of the mammalian neuromuscular junction. In the present study we aimed to evaluate, by means of electrophysiological and pharmacological approaches, which type/s of VDCC are involved in transmitter release at newly formed endplates of the mouse neuromuscular junction. A preliminary report of part of these results has been presented in abstract form (Katz, Ferro, Weisz & Uchitel, 1995).

METHODS

Experiments were carried out on the levator auris longus muscle of adult male Swiss mice weighing 20–30 g. At all stages, the animals were cared for in accordance with national guidelines for the humane treatment of laboratory animals, which are as protective as those of the US National Institutes of Health. Animals were anaesthetized with 2% tribromoethanol (0·15 ml (10 g body wt)$^{-1}$, I.P.) and the posterior auricular branch of the facial nerve that supplies the left levator auris was crushed with forceps close to its entry to the caudal part of the muscle. Under these conditions, in muscles studied at 1 to 6–8 days after surgery, functional synaptic transmission was absent (lack of muscle contraction upon stimulation and absence of evoked endplate potentials or miniature potentials). Normal and denervated animals at different stages after restoration of functional transmission were anaesthetized and immediately exsanguinated. The left muscle with its nerve supply was excised and dissected on a Sylgard-coated Petri dish containing normal Ringer solution (mM): NaCl, 137; KCl, 5; $CaCl_2$, 2; $MgSO_4$, 1; $NaHCO_3$, 12; Na_2HPO_4, 1; glucose, 11; continuously bubbled with 95% O_2–5% CO_2. The preparation was then transferred to the recording chamber to which the different working solutions and drugs were supplied. All experiments were performed at room temperature (19–23 °C).

Evoked endplate potentials (EPPs) and miniature endplate potentials (MEPPs) were recorded intracellularly with conventional glass microelectrodes filled with 3 M KCl (10–15 MΩ resistance). The mean quantal content of the evoked response was evaluated by the coefficient of variation method assuming that EPP amplitudes in normal Ringer solutions follow a Poisson distribution if the preparations are curarized so as to avoid non-linear summation of EPPs (for review and justification of the use of this method in curarized preparations incubated in normal Ringer solution see Martin, 1966 and Hubbard, Llinás & Quastel, 1969). The mean quantal content (m) was calculated as:

$$m = (V_{EPP})^2/((S_{EPP})^2 - (S_{noise})^2),$$

where V_{EPP} is the mean amplitude of the EPP, and S_{EPP} and S_{noise} are the standard deviations of the recorded EPP amplitudes and of the noise, respectively (see Hubbard *et al.* 1969).

However, transmitter release in normal Ringer solution may still follow a binomial rather than a Poisson distribution. If this were the case, m would be overestimated by the coefficient of variation method, above all in the cases of high quantal output (for review see Martin, 1966).

Muscle contraction was prevented by 1·2–1·6 or 0·4–1·2 μM *d*-tubocurarine (dTC) in normal and reinnervating muscles, respectively. After impalement of a muscle fibre, the nerve was continuously stimulated for 1 min at 0·5 Hz and then 50–100 successive EPPs were recorded (minimum 5–10 fibres per muscle). The resting membrane potential of the muscle fibres (V_m) ranged from −65 to −85 mV and from −55 to −75 mV in normal and reinnervating muscles, respectively. Records were rejected if the membrane potential, V_m, was < -55 mV or fell by more than 5 mV during the recording period or if the 10–90% EPP rise time was > 1 ms. For the early reinnervating muscles (8–10 days after denervation), EPPs with rise times of up to 1·4 ms were accepted. No corrections for non-linear summation were made, as dTC concentration was adjusted to obtain EPPs of less than 4 mV (McLachlan & Martin, 1981). The latency of the evoked response was computed as the time elapsed from the stimulation artifact to the foot of the EPP.

For evaluating MEPP amplitude and frequency, MEPPs were recorded for periods of 1–10 min and stored on tape for further analysis. K^+-evoked MEPP frequency was always evaluated after 30 min of incubating the muscle with a modified Ringer solution of the following composition (mM): NaCl, 137; KCl, 10–20; $CaCl_2$, 2; $MgSO_4$, 1; $NaHCO_3$, 12; Na_2HPO_4, 1; continuously bubbled with 95% O_2–5% CO_2.

Presynaptic currents were recorded extracellularly by the perineurial technique (Mallart, 1985). Upon stimulation of the

motor nerve, the preterminal 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. In all cases, upward deflections signal positivity at the recording electrode. In order to avoid any postsynaptic contribution to the records, perineurial currents were assayed in the presence of 20 μM (reinnervating muscles) or 50 μM (normal muscles) dTC.

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 (3·5% agar in 137 mM NaCl) was used as reference. The signals were digitized (TL-1 DMA interface; Axon Instruments), stored and computer analysed. The software pCLAMP 5.5 (Axon Instruments) was used for data acquisition and analysis.

Values are expressed as means ± S.E.M. unless otherwise indicated. Statistical significance (P values in the text and figure legends) was evaluated by the two-tailed Welch's t test (for unpaired values and not assuming equal variances).

Chemicals

Tribromoethanol was purchased from Aldrich. Nitrendipine, was purchased from Research Biochemicals Inc. d-Tubocurarine (dTc), bovine serum albumin (BSA), and all other salts and reagents used were of analytical grade and purchased from Sigma. The synthetic polypeptide ω-conotoxin GVIA (ω-CgTX) was purchased from Peptides International Co. (Louisville, KY, USA). The synthetic polypeptide ω-agatoxin IVA (ω-AgaIVA) was a generous gift from Dr N. Saccomano (Pfizer Inc., Grotton, CT, USA).

Nitrendipine was made up as a 50 mM stock solution in ethanol, stored at 4 °C and protected from light. Experiments in the presence of nitrendipine were carried out in the absence of direct illumination to the preparation except for the time required to position the recording electrode. Working solutions were made a few minutes before use and the final ethanol concentration in control and drug-treated preparations was 0·1% (v/v). In control experiments (not shown), this concentration of ethanol did not affect any of the parameters under study. In the experiments with either ω-CgTX or ω-AgaIVA, both control and toxin-treated fibres were assayed in the presence of a final concentration of 0·01% BSA.

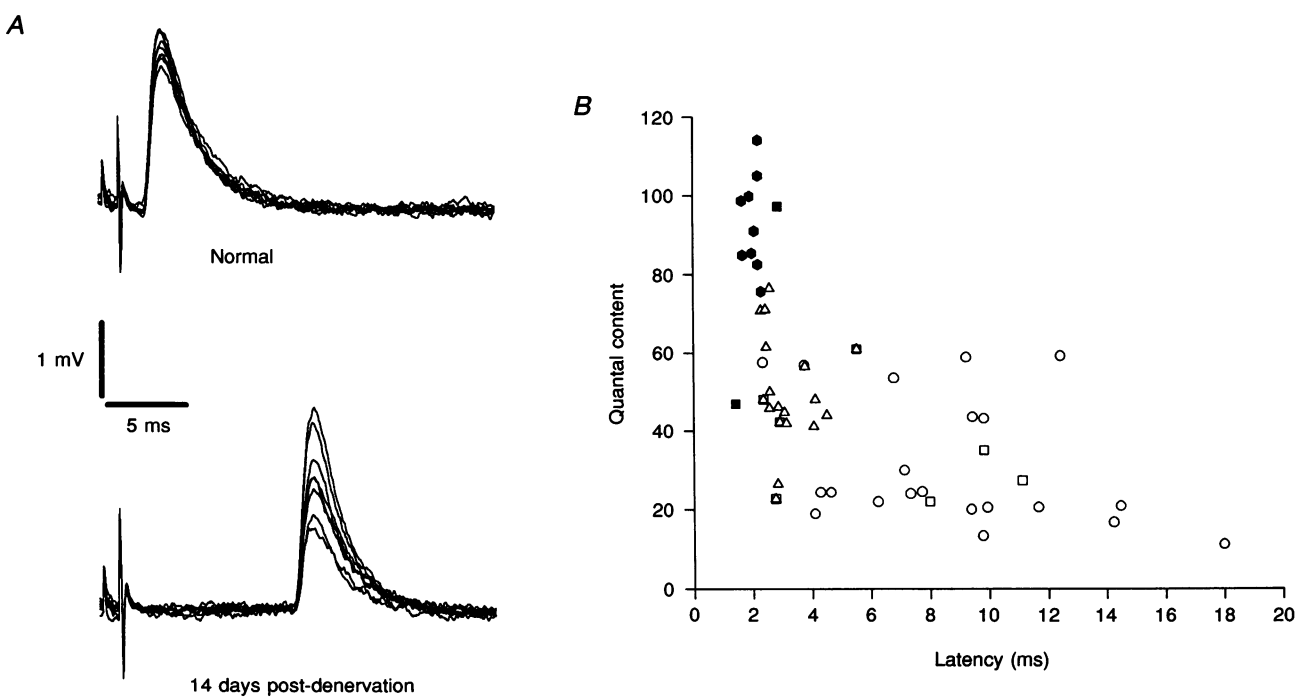

Figure 1. Characteristics of the EPP in the normal and reinnervating levator auris

A, representative recordings of 50 successive stimuli delivered at 0·5 Hz. The upper panel illustrates the type of responses obtained in a normal muscle and the lower panel those obtained in a reinnervating muscle incubated in normal Ringer solution in the presence of 1·3 μM (normal) or 0·6 μM (reinnervating) dTC. *B*, the plot of quantal content, m, *vs.* latency shows that EPPs in reinnervating muscles at early stages post-denervation present much lower m values and longer latency than EPPs in normal muscles. For normal muscles (●) each data point is the mean of 10 fibres (9 muscles), S.E.M. < 10% for m and < 2% for latency. For reinnervating muscles at 62–65 days (■), each data point represents the mean of 14 fibres (2 muscles), S.E.M. < 20% for m and < 4% for latency. For reinnervating muscles at 8–14 days (○; 110 fibres, 14 muscles), 17 days (□; 11 fibres, 1 muscle) and 25–32 days (△; 38 fibres, 4 muscles), each data point represents the mean of 2–7 fibres whose endplates were located at approximately the same distance from the stimulating electrode. For the above muscles, S.E.M. values were 5–30% for m and 2–15% for latency. Standard errors of the means were not plotted for clarity.

RESULTS

Characteristics of the evoked response in the normal and reinnervating levator auris

During the early stages of reinnervation, as shown in other newly formed vertebrate preparations (Bennett *et al.* 1973; Dennis & Miledi, 1974*b*; Tonge, 1974), EPPs presented a lower amplitude, a wider range of amplitude variation during a stimulation train and a longer latency than EPPs in normal muscles (Fig. 1*A*). The estimated quantal contents (m) in normal and reinnervating muscles (Fig. 1*B*) were similar to those reported by Tonge (1974) in curarized mouse preparations. During the course of reinnervation, some muscle fibres with polyneuronal innervation were detected, particularly at < 20 days post-denervation.

Effect of Ca^{2+} channel blockers on nerve-evoked release in normal and reinnervating muscles

In order to evaluate which type or types of VDCC participate in the release process during reinnervation, we tested the effects of specific P/Q-, N- and L-type Ca^{2+} channel blockers on the EPP quantal content. The bar diagram in Fig. 2*A* shows that 30 nM ω-AgaIVA, the P/Q-type VDCC blocker, strongly reduced m values both at normal and newly formed endplates (see also Fig. 3*C*). Higher concentrations (100 nM) of this toxin almost

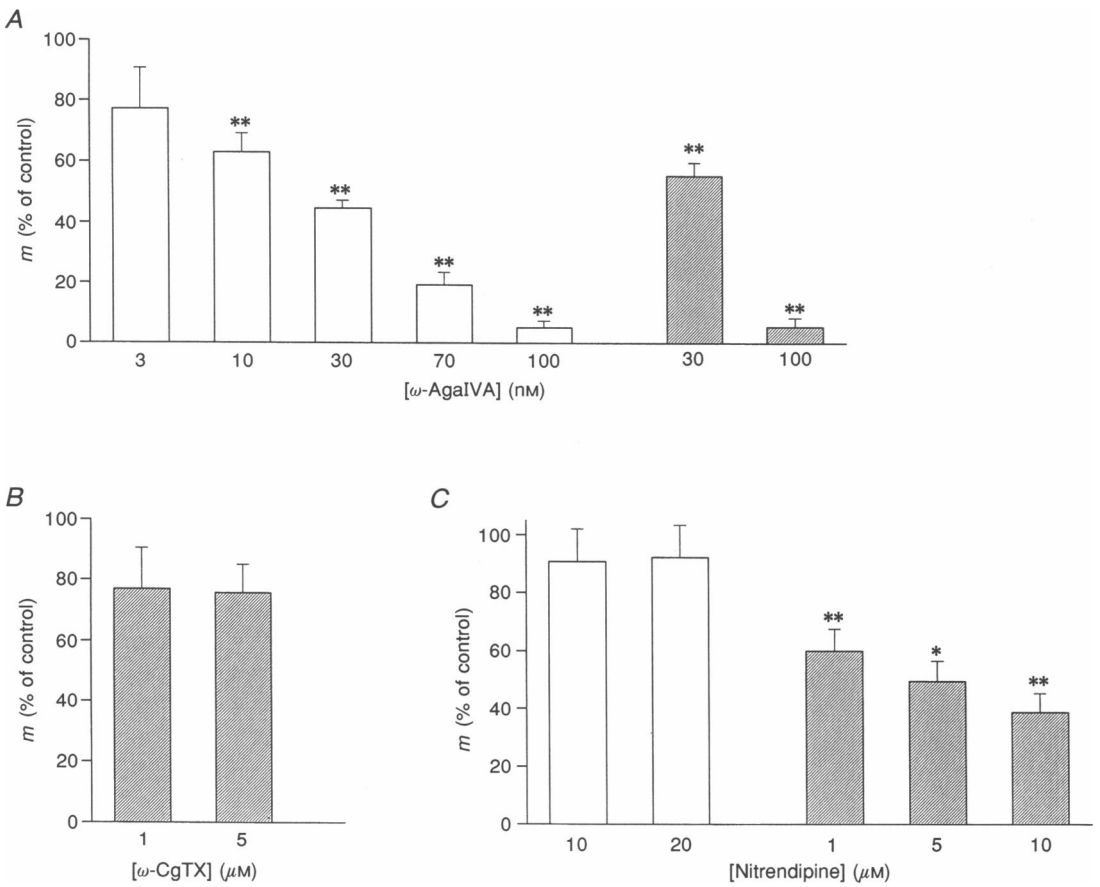

Figure 2. Effect of Ca^{2+} channel blockers on quantal content (m)

A, the bar diagram shows that the P/Q-type channel blocker, ω-AgaIVA reduced m with similar potency in normal (□; control, $m = 90{\cdot}5 \pm 3{\cdot}7$) and reinnervating (▩, 10–17 days post-denervation; control, $m = 37{\cdot}8 \pm 2{\cdot}8$) muscles. The percentage inhibition of m obtained in the normal preparations in the presence of 3, 5, 10, 30, 50, 70 and 100 nM toxin fit to a sigmoidal dose–response curve of the type: percentage inhibition $= \max/(1 + 10^{(\log IC_{50} - \log[\omega\text{-AgaIVA}])})$, where $IC_{50} = 17$ nM, S.D. $= 9$ nM, max $= 106 \pm 11\%$ and $r^2 = 0{\cdot}99$. *B*, the bars show that the N-type channel blocker ω-CgTX did not significantly affect release in reinnervating muscles (control, $m = 28 \pm 2{\cdot}7$). *C*, the bar diagram shows that the L-type channel blocker nitrendipine was able to significantly reduce m in reinnervating muscles (▩; control, $m = 24{\cdot}8 \pm 1{\cdot}7$) and lacked any effect in normal (□; control, $m = 105{\cdot}5 \pm 8{\cdot}5$) preparations. Treated fibres were evaluated after 1 h of incubation with the drug or toxin, in the same muscles in which control fibres were assayed. To increase the signal-to-noise ratio, when blockade was >40% dTC concentration was reduced with respect to that used for controls. Each bar represents the mean ± S.E.M. of at least 10 fibres per muscle (2–6 nerve–muscle preparations). $^{**}P < 0{\cdot}0001$; $^{*}P < 0{\cdot}001$ compared with the values obtained in the same muscles before addition of the VDCC antagonist.

abolished the evoked response in both preparations. The dose-dependent blockade exerted by ω-AgaIVA in the normal levator auris (IC_{50}= 17 nM) is in agreement with what was previously reported in the mouse diaphragm preparation based either on the effects this toxin exerted on m, estimated by the coefficient of variation method (Protti & Uchitel, 1993), or based on its effects on EPP amplitudes (Hong & Chang, 1995). The N-type channel blocker ω-CgTX (1–5 μM), which was reported not to affect neurally evoked synaptic transmission at the mature mouse neuromuscular junction (Protti *et al.* 1991), did not significantly block evoked release at newly formed endplates (Fig. 2*B*). The L-type channel antagonist nitrendipine (1–10 μM), however, caused a significant reduction (~40–60%) in m values at reinnervating endplates (Figs 2*C* and 3*B*) and lacked any effect in normal muscles (Figs 2*C* and 3*A*). This blocker also

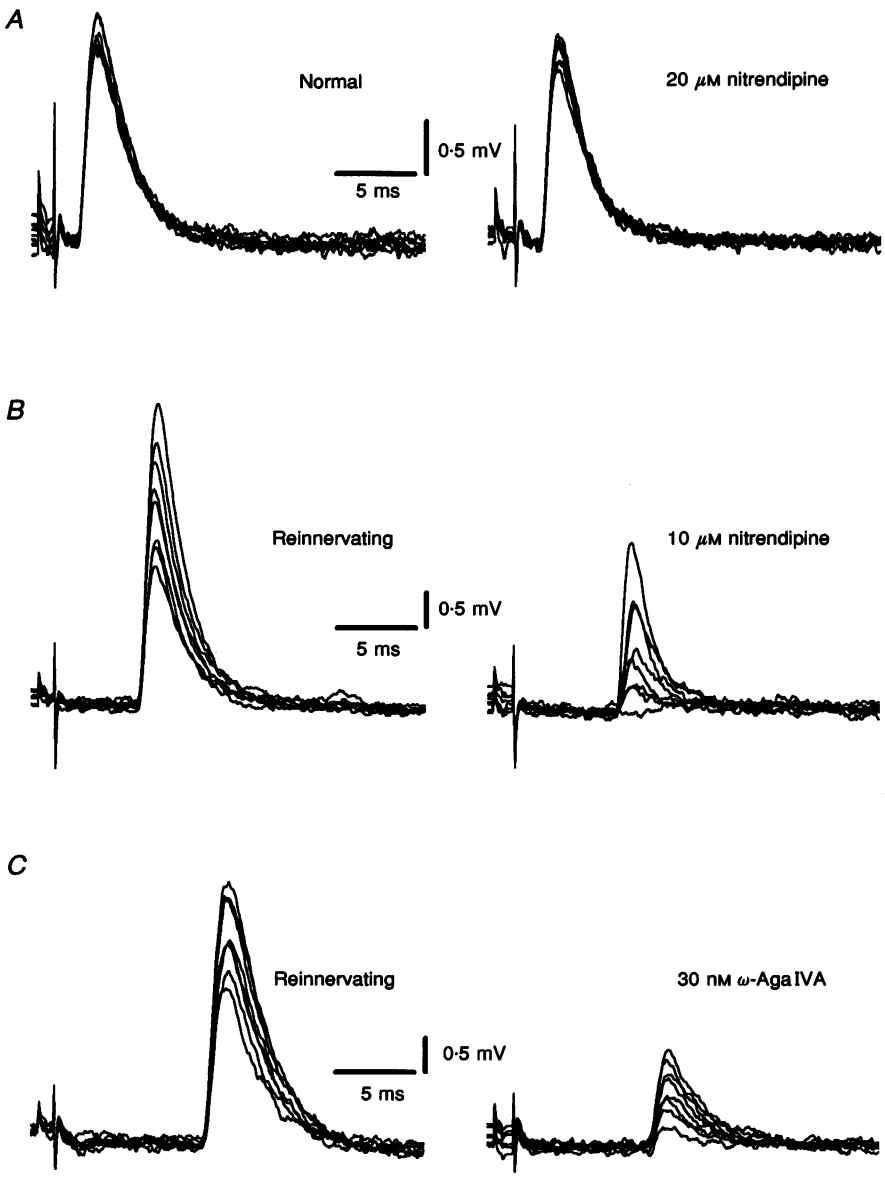

Figure 3. Effect of ω-AgaIVA and nitrendipine on EPP amplitudes

Representative recordings of 50 successive stimuli delivered at 0·5 Hz. *A*, illustrates the lack of effect of nitrendipine on EPP amplitudes in normal muscles. Control, 1·6 μM dTC; drug treated, after 1 h of incubation with 1·6 μM dTC and 20 μM nitrendipine. *B*, illustrates that in reinnervating muscles (EPPs recorded in a muscle 11 days post-denervation), nitrendipine strongly reduced EPP amplitudes and increased their variance, provoking also some release failures. Control, 1·2 μM dTC; drug treated, after 1 h of incubation with 0·2 μM dTC and 10 μM nitrendipine. *C*, illustrates the strong inhibitory effects of low concentrations of ω-AgaIVA in a reinnervating muscle (EPPs recorded in a muscle 14 days post-denervation). Control, 0·8 μM dTC; toxin treated, after 1 h of incubation with 0·4 μM dTC and 30 nM ω-AgaIVA.

lacked significant effects in reinnervating muscles at > 60 days post-denervation (10 μM nitrendipine reduced m to 79 ± 10% of its control value, $P > 0.2$).

As ω-AgaIVA has been shown not to affect MEPP amplitude in normal muscles (Hong & Chang, 1995), the effect of this toxin on transmitter release can be directly assessed by its effects on EPP amplitudes and compared with the blockade based on the estimated m value. In a normal muscle incubated in normal Ringer solution, 30 nM ω-AgaIVA reduced the mean EPP amplitude and the estimated m to 31 ± 5% and 33 ± 5% (11 endplates, 1 muscle) of their control values, respectively. In reinnervating muscles, 30 nM ω-AgaIVA reduced EPP amplitudes and m to 52 ± 7 and 62 ± 10% (15 endplates, 1 muscle at 18 days post-denervation) of their control values, respectively. The effects of nitrendipine on EPP amplitudes were also similar to its effects on m values. Nitrendipine (1 μM) reduced the mean EPP amplitude and m to 42 ± 5 and 49 ± 9% (10 endplates, 1 muscle at 13 days post-denervation) of their control values, respectively.

Effects of ω-AgaIVA and nitrendipine on spontaneous release in reinnervating muscles

At the mature mouse neuromuscular junction, spontaneous release is independent of Ca^{2+} entry through P-type VDCCs, the channel type involved in evoked release (Protti & Uchitel, 1993; Hong & Chang, 1995). It was of interest to study whether at immature endings, the sensitivity of spontaneous release to the action of the VDCC blockers effective in suppressing nerve-evoked release varied with respect to that reported at mature endings. Figure 4*A* shows that neither 100 nM ω-AgaIVA nor 10 μM nitrendipine affected MEPP frequency. These two VDCC antagonists did not affect MEPP frequency in normal levator auris preparations either (data not shown).

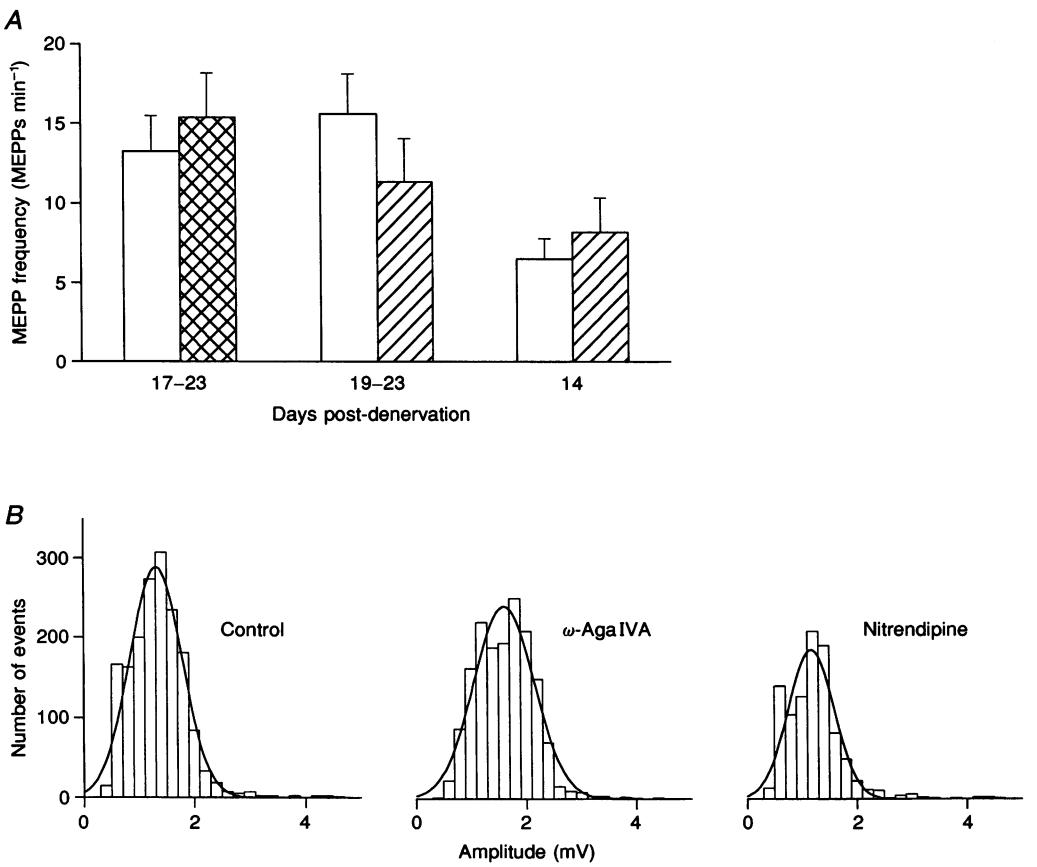

Figure 4. Effects of ω-AgaIVA and nitrendipine on spontaneous release in reinnervating muscles

A, bar diagram showing that MEPP frequency (□, control) was not significantly altered after incubation with either ω-AgaIVA (100 nM; ▩) or nitrendipine (10 μM; ▨). Each bar represents the mean ± S.E.M. of at least 15 fibres per muscle (1–2 muscles). *B*, MEPP amplitude distribution histograms obtained in reinnervating muscles at 17–23 days post-denervation. The histograms represent the amplitude distribution of MEPPs (100 events per fibre) before (control, $n = 4$ muscles) and after 1 h of incubation with either 100 nM ω-AgaIVA ($n = 2$ muscles) or 10 μM nitrendipine ($n = 2$ muscles). There were a few events with amplitudes greater than 5 mV which were not included in the analysis. The histograms were fitted to a Gaussian distribution. Mean amplitudes ± S.D. were: control, 1·3 ± 0·5 ($r^2 = 0.97$); ω-AgaIVA, 1·6 ± 0·5 ($r^2 = 0.95$); and nitrendipine, 1·2 ± 0·4 mV ($r^2 = 0.91$).

Another question regarding the effects of ω-AgaIVA and nitrendipine at newly formed endplates, was whether their impact on EPP amplitudes could be reflecting an effect on the quantal size or on the postsynaptic sensitivity to acetylcholine. As shown in the histograms of Fig. 4*B*, neither the mean amplitude nor the amplitude distribution of MEPPs was affected by ω-AgaIVA (100 nM) or by nitrendipine (10 μM).

Effects of ω-AgaIVA and nitrendipine on K^+-evoked release in reinnervating muscles

A widely employed method for evaluating the types of VDCC present at synaptic terminal membranes, and which does not involve a propagating action potential, is to induce secretion by elevating $[K^+]_o$. The resultant depolarization provokes a considerable increase in MEPP frequency which is dependent on Ca^{2+} entry from the extracellular space (del Castillo & Katz, 1954). As already reported for regenerating vertebrate preparations (Dennis & Miledi, 1974*a*), MEPP frequency was greatly enhanced by incubating the muscles in normal Ringer solution containing 12 and 20 mM K^+ (Fig. 5*A* and *B*). In these conditions, 100 nM ω-AgaIVA, as reported in mature preparations (Protti & Uchitel, 1993), was able to suppress the K^+-evoked increase in MEPP frequency (Fig. 5*A*). Nitrendipine (10 μM), however, was ineffective in antagonizing this type of release (Fig. 5*B*). In normal preparations, nitrendipine was also ineffective (10 mM K_o^+, 407 ± 36 MEPPs min^{-1}; 10 mM K_o^+ + 10 μM nitrendipine, 384 ± 80 MEPPs min^{-1}, $P > 0.7$, $n = 18$ endplates, 1 muscle).

Effect of nitrendipine on the latency of the EPP

In reinnervating muscles, high concentrations of nitrendipine (5 and 10 μM), apart from reducing nerve-evoked transmitter release, provoked a considerable increase in the latency of the EPP. This contrasts with the lack of effect of ω-AgaIVA on this parameter, even at doses that reduced EPP amplitudes and *m* by > 90% (Fig. 6*A*). Figure 6*B* shows that nitrendipine was the only VDCC antagonist that affected the latency of the EPP.

The effect of nitrendipine on the latency of the EPP seems to be independent of the effect this drug exerts on *m*. In reinnervating muscles, 1 μM nitrendipine reduced evoked release (see Fig. 2*C*) without significantly affecting the latency of the EPP (percentage increase, 8 ± 6%, $P > 0.4$). In normal muscles, 10 μM nitrendipine increased the latency of the EPP by 14 ± 2% ($P < 0.001$) without having any effect on *m*. A similar lack of correlation between both effects was found in the late reinnervating muscles (> 60 days) in which 10 μM nitrendipine increased the latency of the EPP by 32 ± 8% ($P < 0.01$) without significantly affecting *m*.

Effect of nitrendipine on perineurial currents

As the observed increase in the latency of the EPP could arise due to a diminution in conduction velocity, the following experiments were designed to test whether nitrendipine could be affecting the currents along nerve branches. As illustrated in Fig. 7, nitrendipine (10 μM) provoked an increase in the latency of the preterminal current elicited at reinnervating nerve branches (Fig. 7*B*) and lacked any detectable effect on this parameter at the mature endings (Fig. 7*A*). This increase in latency of ~0·3 ms, which was always observed at reinnervating endplates upon incubation with nitrendipine, was accompanied by varying degrees of reductions in the amplitude of the signal. Small variations in amplitude, however, could be in part attributed to mechanical artifacts.

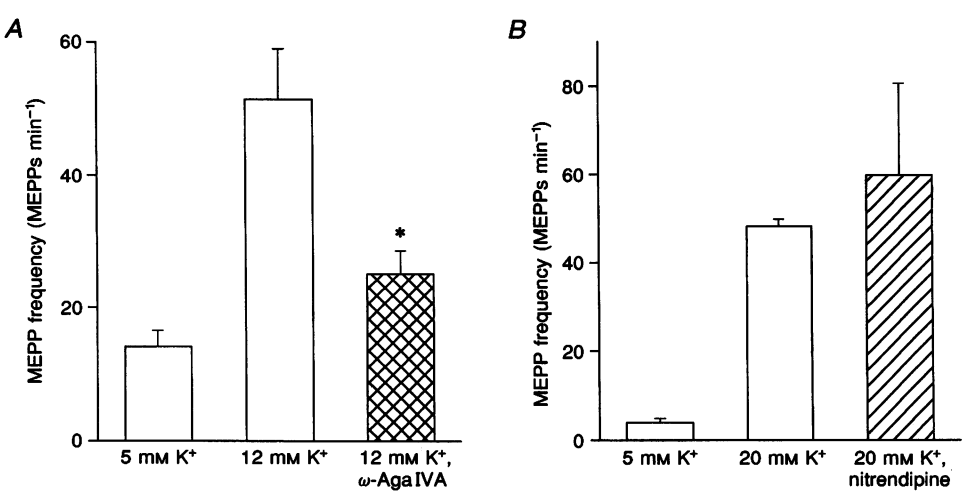

Figure 5. Effects of ω-AgaIVA and nitrendipine on K^+-evoked release in reinnervating muscles

A, the bar diagram illustrates the blockade exerted by 100 nM ω-AgaIVA on the K^+-evoked increase in MEPP frequency. *B*, the bar diagram shows that 10 μM nitrendipine did not affect K^+-evoked release. Treated fibres were evaluated after at least 1 h of incubation with either blocker. Each bar represents the mean ± S.E.M. of at least 15 fibres per muscle (2–3 muscle preparations at 10–14 days post-denervation). *$P < 0.002$ compared with the value in 12 mM K^+ without ω-AgaIVA.

DISCUSSION

The results presented here show that the P/Q-type VDCC blocker, ω-AgaIVA, is able to suppress neurally evoked release at newly formed synapses of the mouse neuromuscular junction. The sensitivity of transmitter release to the action of this toxin is in the same range (30 and 100 nM caused ~50 and 95% blockade, respectively) as that found in normal muscles (IC_{50} = 17 nM; 100 nM caused ~95% blockade) and in agreement with previous results in rodents (Protti & Uchitel, 1993; Hong & Chang, 1995; Wessler *et al.* 1995) and humans (Protti, Reisin, Angelillo Mackinley & Uchitel, 1996). It was found that ω-CgTX does not significantly affect the nerve-evoked response in reinnervating muscles. This indicates that N-type channels, as occur at mature endplates of mice (Protti *et al.* 1991; Bowersox *et al.* 1995), do not contribute to this type of release. By contrast, the L-type channel blocker nitrendipine was found to significantly inhibit nerve-evoked release in reinnervating muscles (1 μM caused ~40% blockade) while being totally ineffective in normal muscles. This lack of effect of dihydropyridines in mature preparations has been previously reported (Penner & Dreyer, 1986; Atchinson, 1989). The reduction of the evoked response by the L-type VDCC blocker in reinnervating endplates is in agreement with previous reports about the involvement of L-type channels in transmitter release at developing neuromuscular synapses (Gray *et al.* 1992; Fu & Huang, 1994).

Neither ω-AgaIVA nor nitrendipine affected spontaneous MEPP frequency or amplitude at reinnervating synapses. This indicates that spontaneous release, as occurs at mature synapses, is not dependent on Ca^{2+} entry through P-type (Protti & Uchitel, 1993) or L-type (Atchinson, 1989) VDCCs. The lack of effect of either ω-AgaIVA or nitrendipine on MEPP amplitude discounts the possibility that the impact of these blockers on EPP amplitudes could be in part accounted for by changes in the quantal size or by a postsynaptic effect.

These results suggest that both P- and L-type channels are involved in controlling nerve-evoked transmitter release at reinnervating synapses. The blockade of P-type channels, however, is enough to completely abolish synaptic transmission. Non-specific effects of nitrendipine on P-type currents do not seem likely owing to the ineffectiveness of

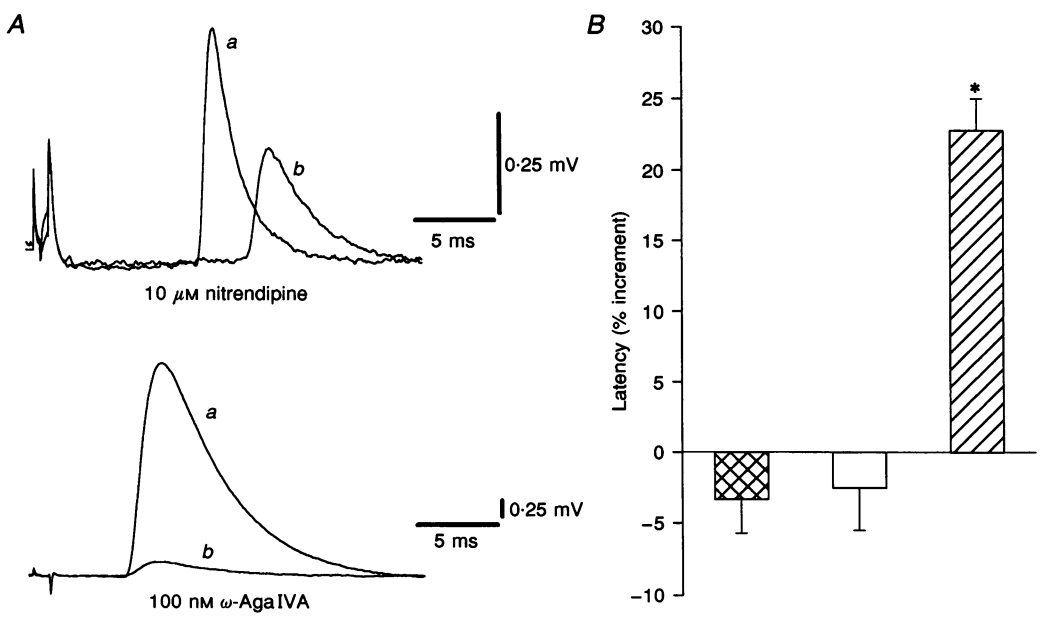

Figure 6. Effect of nitrendipine on the latency of the EPP

A, average of 50 EPPs elicited at 0·5 Hz in reinnervating muscles 14 days after denervation. Upper panel shows the EPPs recorded in the same muscle fibre before (*a*) and after (*b*) 40 min of incubation with 10 μM nitrendipine. In trace *a*, V_m = −58 mV, amplitude = 0·6 mV, m = 21·6, latency = 10·1 ms; in trace *b*, V_m = −62 mV, amplitude = 0·3 mV, m = 8·3, latency = 13·2 ms. Lower panel shows the average of 50 EPPs recorded in the same muscle fibre before (*a*) and after (*b*) 15 min of incubation with 100 nM ω-AgaIVA. In trace *a*, V_m = −62 mV, amplitude = 3·2 mV, m = 38·3, latency = 5·1 ms; in trace *b*, V_m = −62 mV, amplitude = 0·24 mV, m = 2·0, latency = 5·2 ms. The concentration of dTC was not changed upon application of either ω-AgaIVA or nitrendipine. *B*, effects of Ca^{2+} channel blockers on the latency of the EPP. Data are expressed as percentage change in latency with respect to the value obtained in the same muscles before addition of the drug or toxin. Treated fibres were evaluated after at least 1 h of incubation with the drug or toxin. Bar diagrams represent the mean ± S.E.M. obtained in: 60 fibres from 6 muscles at 8–17 days (30 nM ω-AgaIVA; ▩); 12 fibres from 1 muscle at 12 days (5 μM ω-CgTX; □); and 58 fibres, 9 muscles at 11–32 days post-denervation (10 μM nitrendipine; ▨). * $P < 0·0001$ compared with the values obtained in the same muscles before the addition of nitrendipine.

this drug in blocking nerve-evoked release in normal muscles even at high concentrations (10–20 μM). One possible explanation for the overlapping effects of ω-AgaIVA and nitrendipine could be that the population of channels involved in nerve-evoked release at reinnervating motor terminals bears a mixed pharmacological profile. A population of ω-AgaIVA-sensitive channels with a pharmacological profile not consistent with the P-type due to its sensitivity to both dihydropyridines and ω-CgTX has been described in cerebellar granule cells (Pearson, Sutton, Scott & Dolphin, 1995). However, considering the non-linearity of the relationship between $[Ca^{2+}]_0$ and transmitter release (Dodge & Rahamimoff, 1967; Dennis & Miledi, 1974*b*), the overlapping effects of the VDCC blockers on transmitter release cannot simply be accounted for by an overlapping effect on the calcium currents. Another possibility is that at these newly formed synapses, Ca^{2+} entering through more than one type of channel is necessary to trigger secretion at each single release site, as was recently described in a mammalian cerebellar synapse (Mintz, Sabatini & Regehr, 1995).

The effects of high concentrations of nitrendipine on the latency of both the evoked response and perineurial currents suggest that nitrendipine-sensitive channels might be involved in the generation of the nerve action potential along regenerating nerve branches and presynaptic terminals. The blockade of these channels may account for the observed increase in latency, and probably also for the reduction in amplitude, of the perineurial currents. This in turn might affect the amplitude and/or duration of the presynaptic depolarization and consequently modify the amount of transmitter released per impulse (Katz & Miledi, 1970; Augustine, 1990).

There is evidence that nitrendipine, apart from blocking L-type VDCC, reduces the current through Na^+ channels (Yatani & Brown, 1985). However, in normal muscles, neither evoked release nor the perineurial currents were affected by this drug. The lack of effect of high concentrations of 1,4-dihydropyridines on mouse presynaptic currents has already been reported (Penner & Dryer, 1986). Notwithstanding, considering that nitrendipine (10 μM) provoked a small but significant increase in the latency of the EPP in normal muscles, the lack of effect on transmitter release could arise due to the different distribution and proportion of ionic channels along the axons and terminal branches of normal (Mallart, 1985) and regenerating nerves

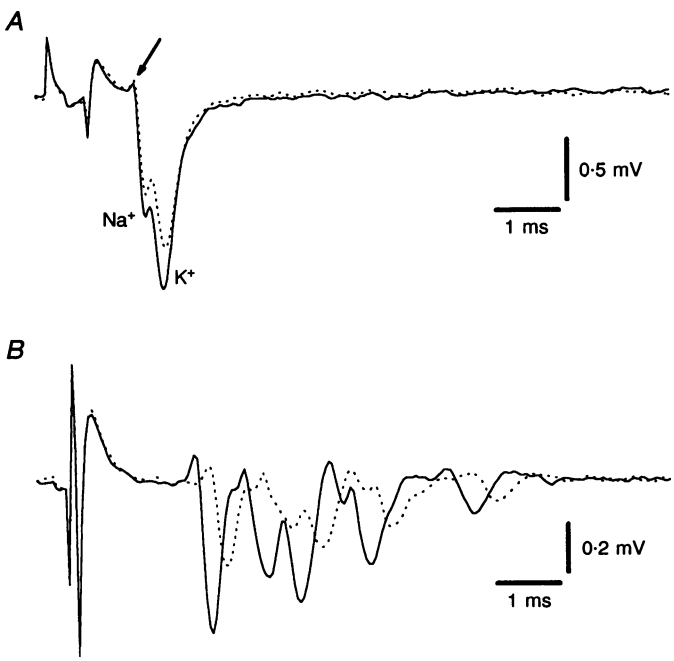

Figure 7. Effects of nitrendipine on perineurial currents

A, a representative recording of the perineurial currents obtained in a normal muscle incubated in normal Ringer solution with 50 μM dTC (continuous line) and after 50 min of adding nitrendipine (10 μM) to the bath solution (dotted line). Arrow indicates local circuit currents associated with the propagating action potential, Na^+ indicates sodium inward current at the recording site and K^+ indicates the sum of outward potassium and inward calcium currents at the terminal membrane. Note that nitrendipine provoked no detectable effects on the latency of the signal. *B*, a representative recording of the perineurial currents obtained in reinnervating muscles (14–17 days post-denervation) incubated in normal Ringer solution with 20 μM dTC (continuous line) and after 60 min of adding nitrendipine (10 μM) to the bath solution (dotted line). Note that nitrendipine provoked an increase in the latency of the signals accompanied by a significant reduction in amplitude. All traces are the average of 10 successive responses elicited at 0·5 Hz. Stimulation artifacts were reduced for clarity.

(Bostock *et al.* 1981; Angaut-Petit & Mallart, 1985*a*, *b*). If nitrendipine were blocking a given fraction of Na^+ channels along the nerve branches and presynaptic terminals in both normal and reinnervating preparations, this fraction could be negligible in terms of transmitter release in normal preparations but could become important along regenerating nerves where the safety factor for conduction of an impulse to the presynaptic terminal is greatly diminished (Dennis & Miledi, 1974*a*). Moreover, at these immature synaptic terminals the coupling between presynaptic depolarization and transmitter release could be less efficient.

It is well known that during development, action potentials both in the neuronal somata and neurites of several systems have a predominant Ca^{2+} component (reviewed by Spitzer, 1994). In non-myelinated axons from rat dorsal root ganglion neurons in culture, it has recently been demonstrated that Ca^{2+} entering through channels present in the axons plays an important role in regulating action potential propagation (Lüscher, Lipp, Lüscher & Niggli, 1996). Based on the effects of dihydropyridines, the entry of Ca^{2+} through L-type channels has been implicated, during both development and reinnervation of the vertebrate neuromuscular junction, in processes such as axonal sprouting after damage to the motor nerve (Angelov, Neiss, Streppel, Andermahr, Mader & Stennert, 1996) and neurite retraction during the elimination of polyneuronal innervation (Zhu & Vrbová, 1992). Accordingly, the present results also suggest that L-type calcium channels might be present along regenerating neurites.

Another finding that suggests that L-type channels might be involved in presynaptic depolarization and be located further away from the release sites than P-type channels seem to be, is that ω-AgaIVA is able to impair both nerve- and K^+-evoked release, whereas nitrendipine is only effective in reducing nerve-evoked release. The VDCC involved in K^+-induced release, however, might be different from those operating during nerve-evoked depolarization (Momiyama & Takahashi, 1994). Besides, the level of depolarization provoked by elevating external $[K^+]$ to 20 mM might not have been sufficient to obtain a significant contribution of L-type channels to this type of release.

In conclusion, the results show that at newly formed synapses of the mouse neuromuscular junction, as at mature synapses, channels of the P-type family play a prominent role in triggering evoked release whereas N-type channels are not involved in this process. In addition, a relevant difference found between immature and mature synapses is that both action potential propagation and transmitter release become highly sensitive to nitrendipine during reinnervation. This suggests that L-type channels may be involved in the control of synaptic transmission at immature synapses of the mammalian neuromuscular junction.

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