Transmitter-mediated local contracture of the endplate region of the focally innervated mouse diaphragm treated with anticholinesterase

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1 Local contraction of the endplate region in response to nerve stimulation was studied in isolated mouse diaphragms. In normal preparations, muscle contractions involved the whole length of the muscle fibre with rise and decay times in the order of tens of ms whether evoked with a single or train of pulses.

2 When acetylcholinesterase was inhibited with neostigmine, tetanic stimulation produced a twitch-like phasic contraction and a delayed tonic contracture. A brief train of pulse (10 ms, 300 Hz) was enough to trigger a full size tonic contracture which reached an amplitude about one tenth that of control tetanus and had a duration of about 4 s.

3 Tetanic stimulation evoked a non-propagating prolonged depolarization at the endplate region lasting for about 1 s following a few muscle action potentials.

4 μ -Conotoxin, a specific inhibitor of muscle Na^+ channel, selectively abolished the phasic contraction and the muscle action potentials leaving the tonic contracture and the prolonged depolarization unaffected.

5 Both the tonic contracture and the prolonged depolarization were highly sensitive to blockade by tubocurarine (IC_{50} 0.05–0.1 μM) and vesamicol (1 μM , an inhibitor of packaging acetylcholine into synaptic vesicles), were attenuated by increasing Ca^{2+} concentration and were prolonged by decreasing Ca^{2+} .

6 The results suggest that prolonged activation of endplate nicotinic receptors by endogenously released transmitter can produce substantial contractions of the endplate region when acetylcholinesterases are inhibited. The source of Ca^{2+} for the contraction seems to come mainly from intracellular stores.

Keywords: Anticholinesterase; muscle contracture; neuromuscular junction

Introduction

The physiological process that triggers excitation-contraction coupling in skeletal muscle is depolarization of the sarcolemma (Rios & Pizarro, 1991). For the multiply innervated muscle fibres, as found in amphibia, birds, fish and extraocular muscles of mammals, which have high densities of innervations and widespread distribution of acetylcholine (ACh) receptors, the whole muscle membrane can be directly depolarized by the transmitter released from adjacent nerve terminals and the resulting depolarization is sufficient to activate the contractile mechanism (cf. Bowman, 1990). On the other hand, most mammalian muscle fibres are focally innervated and the depolarization of the whole plasma membrane relies on the propagative action potential that is initiated by the junctional endplate potential (e.p.p.) which is confined to the endplate region. The question then arises whether e.p.p. produces a local contraction of the motor endplate region of the focally innervated muscle fibre. Burd & Ferry (1987) reported a contraction of the endplate region after a single indirect stimulation when treated with anticholinesterase. In the present study, we explored the direct effects of e.p.p. on the contraction of the endplate region of mouse diaphragm, a focally innervated striated muscle, by deliberately abolishing the muscle action potential with μ -conotoxin, a specific blocker of the muscle Na^+ channel (Gray & Olivera, 1988; Hong & Chang, 1989b). We investigated in particular the effect of repetitive stimulation on the

contraction of the endplate region when the hydrolysis of ACh by acetylcholinesterase is retarded since under such a condition the amplitude and duration of the e.p.ps are increased (Katz & Miledi, 1975) and the regeneratively released neurotransmitter depolarizes endplate region for a prolonged period of time (Chang & Hong, 1986; Hong & Chang, 1989a).

Methods

Phrenic nerve-hemidiaphragm preparations were isolated from mice (ICR strain, 25–30 g). The organ chamber contained 15 ml Tyrode solution (composition in mM: NaCl 137, KCl 2.8, CaCl_2 1.8, MgCl_2 1.1, NaHCO_3 11.9, NaH_2PO_4 0.33 and dextrose 11.2) unless otherwise indicated. The solution was maintained at 34–36°C and the pH adjusted to 7.2–7.4 by aerating with a gas mixture of 5% CO_2 in O_2 . Preparations were loaded with 1–1.5 g resting tension. Indirect contractions were evoked by stimulation of the trunk of the phrenic nerve with supramaximal pulses of 0.05 ms duration and direct contractions by field stimulation with 0.5 ms duration unless otherwise indicated. Tetanic contractions were elicited by a train of pulses consisting of variable number of pulses (2–600) at different frequencies (25–300 Hz). The interval between trains of stimulation was 10–15 min to minimize post-tetanic influences. Mechanical responses were recorded with a sensitive isometric transducer (Gould BG25), which can differentiate a small change of tension (0.03 g) from environmental vibrations. The contraction of the diaphragm was also visualized through a stereomicroscope, equipped with a plan or achromat objective (Zeiss SV 8).

Three procedures were used to paralyze diaphragms for

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electrophysiological studies. (1) Preparations were treated with μ -conotoxin (0.75–1.25 μ M). (2) Formamide, a chemical that modifies the function of sarcoplasmic reticulum (Del Castillo & Escalona De Motta, 1978) was used. The nerve-muscle preparations were incubated in Tyrode solution containing 2–3 M formamide under direct stimulation at 2 Hz for 25–35 min and thereafter, equilibrated in normal Tyrode solution for 30–60 min. (3) In some experiments, cut muscle preparations were used. Both ends of diaphragm muscles were crushed with forceps and left standing in Tyrode solution for 1.5–2 h until the resting membrane potential declined from about -80 mV to -40 to -46 mV. Immobilizations were considered adequate if the developed tension in response to a test train of direct stimulation (200 Hz, 3 s) was reduced from a control level of 15–20 g to less than 0.3 g. Membrane potentials, e.p.ps and miniature e.p.ps were recorded conventionally with a high impedance amplifier (Dagan 8100). Glass microelectrodes filled with 3 M KCl had resistance of 5–15 M Ω . The signals were filtered at 10 kHz and digitized at 100 kHz and recorded on an electrostatic recorder (Gould ES 2000). Membrane potentials in each preparation from 25–30 muscle fibres were pooled excluding the lowest 10% of the values. The microelectrode was assumed to be targeted at the endplate region when the rise time of the sampled miniature e.p.p. was less than 0.6 ms (without treatment of neostigmine) or 1 ms (after neostigmine). The quantal releases of e.p.ps were estimated by direct comparisons of e.p.p./miniature e.p.p. in preparations paralyzed with μ -conotoxin, in which e.p.ps (evoked at a rate of 0.7 Hz) and miniature e.p.ps were sampled from the same junction for 35 s. Both amplitudes of e.p.ps and miniature e.p.ps were corrected for non-linear summation with a reference membrane potential of -80 mV as previously described (Chang *et al.*, 1986).

The duration of contraction or endplate depolarization was defined as the time spanning the time course of 30% peak amplitude. The rise and decay times of contraction were defined as the time for 10 to 90% and from 90 to 10% peak amplitude, respectively. All data are presented as means $\pm$ standard error of recordings obtained from at least 4 preparations. Differences between means were analyzed by Student's *t* test and a statistical *P* value less than 0.05 was considered significant.

Chemicals used were neostigmine, tetrodotoxin and tubocurarine (Sigma Chem. Co., St. Louis, U.S.A.). μ -Conotoxin was purchased from Peptide Institute (Osaka, Japan). Vesamicol (AH5183) was generously supplied by Glaxo (London, U.K.).

Results

Effects of neostigmine on muscle contraction

In control preparations, diaphragms contracted and relaxed rapidly in response to single direct or indirect stimulations with the following parameters: amplitude 1.6 ± 0.2 g, rise time 14 ± 1 ms, decay time 34 ± 3 ms, and duration 36 ± 3 ms ($n = 5$). In order to produce a well-summed tetanic contraction, repetitive pulses greater than 75 Hz were required; otherwise the contractile force was far less than maximum and had a ripple contour (Figure 1). At a stimulation frequency of 200–300 Hz, the amplitude of tetanic contraction approached maximum after about 10 pulses of stimulation and the developed force was nearly 10 fold that of single twitch. On termination of stimuli, the tetanic tension decayed to the pre-stimulation basal level almost as rapidly as a single twitch (Figure 1a).

After treatment with neostigmine at 0.2 μ M, a concentration sufficient to provide substantial inhibition of acetylcholinesterase but not to produce side effects on the ACh receptor ion channel (Chang *et al.*, 1987), the direct twitch

Figure 1 Effects of pulse number and stimulation frequency on indirect contraction of mouse phrenic nerve-diaphragm. (a) Control contractile responses showing single twitch (a1) and tetanic contractions for 0.6 s evoked at 50 Hz (a2), 100 Hz (a3) and 200 Hz (a4). Calibrations: 0.3 s and 5 g. (b) Contractile responses of the same preparation after 30 min incubation with 0.2 μ M neostigmine showing the spontaneous fasciculation and the evoked response after a single pulse (arrowhead, b1) and the evoked responses to train of pulses at 50 Hz for 0.6 s (b2) and at 200 Hz for 0.03 s (b3) or for 3 s (b4). Calibrations: 0.3 s (b1), 6 s (b2–b4) and 5 g. Note in (b) the tonic contractures of equal amplitude and duration irrespective of pulse frequency or length of train of pulses.

force was potentiated 4–5 fold (7.8 ± 0.9 g) and the rise time of twitches was approximately doubled (26 ± 2 ms). All of these changes can be ascribed to the repetitive firings of muscle action potentials in response to a single indirect stimulation (cf. Hobbiger, 1976). The decay time of twitches appeared unchanged (32 ± 3 ms). When stimulated with a train of pulses at 40–300 Hz, the contraction of the preparations had two distinct components: an initial twitch-like phasic contraction with a fast relaxation (i.e., a tetanic fade) and a late tonic contracture which reached the peak 400–600 ms after the start of the train of pulses (Figure 1b). The amplitude of the phasic contraction was about 70% that of the control tetanic contraction (11.2 ± 0.8 vs 15.7 ± 1.1 g, $n = 8$), i.e., the tetanic contraction waned before reaching the maximal contractility. The amplitude of the tonic contracture (1.7 ± 0.1 g) was about one tenth that of the control maximal tetanus but its duration was more than a hundred times longer than that of the twitches (3924 ± 210 vs 36 ± 3 ms). This late tonic contracture was characterized by a slow rise and slow decay as compared to those of the phasic contraction. Once triggered, both the magnitude and duration of the tonic contracture remained the same at stimulation frequencies ranging from 50 to 300 Hz. The higher the stimulation frequency the fewer the number of pulses were needed such that, at 300 Hz, a 10 ms short train of stimulation consisting of 4 pulses were sufficient to elicit the fullsize tonic contracture. Additional pulses extending over 1000 ms did not change the profile of contracture but increased slightly the tension at decay phase (Figure 1b4 vs 1b3). The tonic contracture could be repeated and the size maintained for 2 h if the interval of tetanic stimulations was longer than 10 min.

However, when train of pulses were repeated at intervals shorter than 5 min the size of tonic contracture declined within 1 h.

Effects of μ -conotoxin and formamide on the phasic and tonic contractions

μ -Conotoxin at a concentration of 0.75–1.25 μ M abolished the indirect as well as direct twitches and tetanic contractions (Figure 2), confirming the blockade of muscle Na^+ channels (Hong & Chang, 1989b). Moreover, a train of indirect stimulations produced no change of light transmittance at the endplate region, indicating that no local contraction is elicited even in the presence of repetitive e.p.s.ps when acetylcholinesterase is not inhibited. After further addition of neostigmine, single indirect stimulations evoked small amplitude twitches (the amplitude was 7–15% that of control, Figure 2) with a slow rise time and a prolonged duration (Figure 3a), referred as slow twitches hereafter. A train of stimulation (at 40–300 Hz) now evoked a full-sized tonic contracture without the initial phasic twitch component (Figure 2a4). During optical examination the transmittance of light around the endplate region, but not the rest of the muscle, was reduced at times when indirect stimulations elicited the tonic contracture, indicating that only the endplate region is involved in the tonic contracture. When added to neostigmine-pretreated preparations, μ -conotoxin inhibited the phasic response completely without changing the tonic contracture in response to trains of stimulation (Figure 2b). The profiles and magnitudes of the control tetanus and the tonic contracture are summarized in Figure 3b. When preparations were immobilized with formamide, neither single nor repetitive indirect stimuli produced observable contraction, no matter whether acetylcholinesterase was inhibited or not.

Effect of tubocurarine on the tonic contracture

If the muscle Na^+ channel was not inhibited, tubocurarine (0.03–0.3 μ M) partially antagonized the tetanic fade pro-

duced by neostigmine when the nerve was stimulated with a train of pulses at 75–100 Hz (Chang *et al.*, 1988; Figure 4a4). By contrast, in preparations paralyzed with μ -conotoxin, tubocurarine dose-dependently inhibited the magnitude of the slow twitches and the tonic contractures and shortened the duration of the contractures (Figure 4 and Table 1). At 0.2 μ M, tubocurarine abolished all indirect contractions. In this respect, tubocurarine was 10 fold more potent than in inhibitions of twitches in normal preparations (IC_{50} : 0.07 ± 0.02 vs 0.86 ± 0.10 μ M, $n = 5$).

Effects of Ca^{2+} on the tonic contracture

The influence of low (0.45 mM) and high (7.2 mM) Ca^{2+} Tyrode solution on the slow twitch and the tonic contracture was studied. Low and high Ca^{2+} Tyrode solution changed the quantal release to 25 ± 4 and $167 \pm 10\%$ of control ($n = 4$), respectively. These changes in Ca^{2+} concentration had negligible effect on direct twitches within 40 min (being $93 \pm 3\%$ of control, $n = 4-8$). The small slow twitches evoked after treatment with neostigmine and μ -conotoxin were abolished in low Ca^{2+} Tyrode solution but were potentiated in high Ca^{2+} Tyrode solution (Figure 5). Interestingly, the effects of Ca^{2+} on the tonic contracture were different. In low Ca^{2+} Tyrode solution though more pulses were needed to evoke the tonic contracture, the contracture had a more prolonged duration than that obtained in normal Tyrode solution. While in high Ca^{2+} Tyrode solution both the duration and the magnitude of the tonic contracture were suppressed (Figure 5, Table 1).

Inhibition of tonic contracture by vesamicol

The effects of modifying ACh release on the prolonged responses were tested in another way by the use of vesamicol, which is a specific inhibitor of the vesicular ACh transport system (Marshall & Parsons, 1987). In preparations treated with neostigmine but without indirect stimulation, treatment with vesamicol at 1 μ M did not change the phasic contraction nor the tonic contracture tested at 2 h of incubation. How-

Figure 2 Effects of μ -conotoxin on direct and indirect contractions of mouse phrenic nerve-diaphragm. (a) The preparation was treated, in sequence, with μ -conotoxin (1.25 μ M) at $\blacktriangle$ and with neostigmine (0.2 μ M) at $\blacktriangleup$. The stimulation modes were: (a1) indirect and direct pulses alternatively at 0.1 Hz; (a2) indirect train of pulse at 300 Hz for 3 s; (a3) indirect pulse at 0.1 Hz, and (a4) indirect train of pulse at 300 Hz for 0.01 s. Calibrations: 1 g and 3 s except in a1 and a3 where the intervals between single twitches were 10 s. (b) Another preparation pretreated with 0.2 μ M neostigmine and stimulated with train of pulses at 50 Hz for 3 s. (b1) Control, and (b2) 15 min after 1.25 μ M μ -conotoxin. Calibrations: 5 g and 3 s. Note that μ -conotoxin isolates the late tonic contracture from the phasic contraction.

Figure 3 Effects of neostigmine and μ -conotoxin on the time course and amplitude of indirect contraction of mouse phrenic nerve-diaphragm. Phrenic nerves were stimulated either with a single (a) or with a train of pulses at 200 Hz for 0.3 s (b). The contractile responses were evoked in the absence or presence of neostigmine ($0.2 \mu\text{M}$) and μ -conotoxin ($1.25 \mu\text{M}$). Four parameters of indirect contraction were measured: $\square$ 10–90% rise time; $\square$ 70% duration; $\square$ 90–10% decay time, and $\blacksquare$ contractile force. Data were pooled from 5–8 preparations.

ever, when the phrenic nerve was periodically stimulated with trains of pulses (100 Hz for 3 s every 10 min for 40 min), vesamicol increased the amplitude of the phasic contraction slightly by $9 \pm 2\%$ but inhibited the tonic contracture by $49 \pm 5\%$ (estimated from depressions of contracture areas, $n = 4$). The increase of the phasic contraction is a common phenomenon also observed on treatment with low concentrations of tubocurarine or low Ca^{2+} Tyrode solution (data not shown) and seems to be due to an inhibition of the induction of the prolonged endplate depolarization and hence to an increased number of initial muscle action potentials.

Figure 4 Effects of tubocurarine and μ -conotoxin on tonic contracture of mouse phrenic nerve-diaphragm treated with neostigmine. Preparations were treated with $0.2 \mu\text{M}$ neostigmine (a) or, in addition, with $1.25 \mu\text{M}$ μ -conotoxin (b). Tubocurarine was added at concentrations of 0, 0.05, 0.1 and $0.2 \mu\text{M}$, respectively, for panels 1, 2, 3 and 4. Phrenic nerves were stimulated first with 6–7 single pulses at 0.1 Hz and then at 200 Hz for 0.1 s, except in panel 4, where stimulation at 75 Hz for 3 s was applied. Calibrations: 1 g and 3 s (for tetanic contraction or tonic contractures). Note that tubocurarine dose-dependently inhibited tonic contractures (a2, a3, b2, b3 and b4) and slow twitches (b2 and b3) while partially restoring the tetanic contraction at 75 Hz (a4).

Effects of membrane potentials of endplate

In formamide-immobilized preparations treated with neostigmine, trains of indirect stimulation produced initially a few muscle action potentials and then a local prolonged

Table 1 Effects of Ca^{2+} , tubocurarine and μ -conotoxin on the depolarization of endplate and the late tonic contracture of mouse phrenic nerve-diaphragm

Treatment ^a	Tonic contracture (%)		Endplate depolarization (%) ^b	
	Amplitude	Duration	Amplitude	Duration
Ca^{2+} (mM)				
0.45	93 ± 4	$121 \pm 5^*$	89 ± 9	$128 \pm 11^*$
1.8 ^c	100	100	100	100
3.6	$79 \pm 7^*$	$72 \pm 8^*$	105 ± 4	$62 \pm 10^*$
7.2	$54 \pm 9^*$	$41 \pm 7^*$	96 ± 6	$30 \pm 3^*$
Tubocurarine (μM)				
0.05	$84 \pm 3^*$	$70 \pm 6^*$	93 ± 3	$49 \pm 8^*$
0.1	$49 \pm 6^*$	$33 \pm 5^*$	$75 \pm 7^*$	$22 \pm 5^*$
μ -Conotoxin (μM)				
1.25	96 ± 4	98 ± 3	98 ± 5	103 ± 4

^aPreparations were incubated in Tyrode solution containing $0.2 \mu\text{M}$ neostigmine and phrenic nerves were stimulated at 200 Hz for 0.1–0.3 s.

^bCut muscle preparations were used and the 70% duration of the prolonged endplate depolarization (6–11 endplates for each preparation) was calculated.

^cThe control values for the amplitude and duration in normal Tyrode solution were 1.6 ± 0.2 g and 3924 ± 210 ms (for the tonic contracture), and 20.1 ± 1.9 mV and 924 ± 67 ms (for the endplate depolarization), respectively.

* $P < 0.05$ vs the values in Tyrode solution containing 1.8 mM Ca^{2+} .

Data were pooled from 4–8 preparations.

Figure 5 Effects of Ca^{2+} on indirect contraction of mouse phrenic nerve-diaphragm treated with neostigmine. Preparations were pretreated with $0.2 \mu\text{M}$ neostigmine (a) or, in addition, with $1.25 \mu\text{M}$ μ -conotoxin (b). Calcium concentrations in Tyrode solution were 0.45, 1.8 and 7.2 mM , respectively, for panels 1, 2 and 3. Phrenic nerves were first stimulated at 0.1 Hz for 6 pulses and then with train of pulse at 200 Hz for 0.1 s. Calibrations: 1 g and 3 s (for tonic contractures). Note changes in amplitude and duration of tonic contractures (a and b) and amplitude of slow twitches (b).

depolarization of the endplate (Figure 6). The action potentials can account for the twitch-like phasic contraction in non-immobilized preparations. The magnitude of depolarization at plateau was about 20–30 mV and the duration lasted up to 1000 ms. Once the prolonged depolarization occurred, continued stimulation of the motor nerve failed to elicit phasic e.p.p., and the stimulations had no apparent effect on the magnitude and duration of the prolonged depolarization as previously reported in cut muscle preparations (Chang & Hong, 1986; Hong & Chang, 1989a). When recorded at a site about 3–4 mm away from the endplate region, only 3–8 muscle action potentials were registered at the beginning of a train of pulse each having a uniform size of amplitude. Thereafter, no residual membrane depolarization was observed. These results demonstrate that the tonic contracture was not associated with muscle action potentials and that the depolarization was confined to the endplate area.

μ -Conotoxin abolished the muscle action potentials but totally preserved the prolonged endplate depolarization (Figure 6). As compared in the figure, the endplate depolarization occurred ahead of the tonic contracture although it was of shorter duration. At a stimulation frequency of 35 Hz, about two thirds of junctions failed to produce the prolonged endplate depolarization. Simultaneously, it was observed that the amplitude as well as the duration of the tonic contracture decreased.

The prolonged depolarization was shortened and inhibited by tubocurarine (Table 1) and by vesamicol ($1 \mu\text{M}$, data not shown) in parallel with the depression of tonic contracture. Low and high Ca^{2+} Tyrode solution affected the duration of the prolonged depolarization in the same way as they both changed the duration of the tonic contracture (Table 1).

Contraction evoked by direct stimulation of muscle

In view of the relationship between tonic contracture and prolonged depolarization, the effect of a long direct electrotonic depolarization of muscle, bypassing activation of

nicotinic receptor and muscle Na^+ channel, was studied in the presence of tubocurarine and tetrodotoxin. An intensive field stimulation of the diaphragm with a rectangular pulse of 10–300 ms duration evoked a prolonged contracture for 2–6 s with a characteristic slow decay (Figure 7). Both the amplitude and duration of the contracture increased as the pulse duration was lengthened. By contrast, the tetanic contractions produced by a train of short pulses (pulse duration 0.5 ms at 300 Hz for 3 s) were of lower amplitude than that induced by a long pulse (100 ms) and relaxed rapidly after termination of stimuli, suggesting that a mechanism for tonic contracture is triggered by a prolonged depolarization but hardly by repetitive short pulses. These contractions were not blocked in Ca^{2+} -free Tyrode solution containing 3 mM EGTA, suggesting that they utilize the Ca^{2+} mobilized from intracellular stores. The changes of muscle membrane potential during such a prolonged contracture were studied in formamide-immobilized diaphragms. The recording micro-electrode was inserted into the portion of muscle fibre close to one of the field stimulation electrodes. After the application of a long pulse (100 ms), the membrane potential remained depolarized by 20–40 mV for 0.4–0.8 s and then repolarized to pre-stimulation level. Clearly, the time course of the pulse-induced contracture outlasted that of the pulse-induced membrane depolarization. This temporal relation between contracture and membrane depolarization is similar to that between the tonic contracture and the prolonged endplate depolarization evoked by indirect stimulations in neostigmine-treated preparations.

Discussion

The present results revealed that, unlike the multiply innervated muscle fibres, the endplate region of the focally innervated fibres do not contract in response to single or trains of high frequency e.p.s in normal condition when the muscle action potential is abolished by μ -conotoxin. This situation, however, is changed and muscles contract in response to indirect stimulation when the hydrolysis of released ACh is retarded. In the presence of neostigmine, single stimuli elicited slow twitches and a train of pulses evoked tonic contractures. Both contractions are not associated with muscle action potential, are resistant to μ -conotoxin but are inhibited by tubocurarine. These results indicate that the contractions are initiated by the endogenous ACh released from the nerve terminal and are confined to the endplate regions of muscle fibres. In the presence of anticholinesterase, the single e.p.p. is not only enhanced but also prolonged (Katz & Miledi, 1975). This may account for the slow twitch responses. Since repetitive stimulation tends to provoke a very marked long-lasting endplate depolarization presumably due to a regenerative release of ACh (Chang & Hong, 1986; Hong & Chang, 1989a), it is likely that the endplate region elicits the tonic contracture in response to the prolonged local depolarization.

The late tonic contracture and the prolonged endplate depolarization observed in neostigmine-treated preparations share several characteristic features. (1) Both phenomena are resistant to μ -conotoxin and are restricted to the endplate area. (2) Both phenomena, once triggered, bear an 'all' property as revealed by the full-size of the tonic contracture and depolarization, irrespective of pulse number and stimulation frequency. (3) The two responses are evoked in parallel by indirect stimulation in terms of stimulation frequency and number of pulses, are modulated in the same way by changes of Ca^{2+} concentration, and show high susceptibilities to blockade by tubocurarine or vesamicol. Moreover, 3,4-diaminopyridine, a K^+ channel blocker also known to produce a prolonged endplate depolarization on indirect stimulation (Hong & Chang, 1990), provoked a similar tonic contracture (unpublished observation), indicating that an inhibition of acetylcholinesterase *per se* is not a prerequisite for such

Figure 6 Temporal correlations of the prolonged endplate depolarization and the tonic contracture of mouse phrenic nerve-diaphragm treated with neostigmine. The electrophysiological tracings (a and b) were taken from the same endplate of a preparation immobilized with formamide and treated with $0.2 \mu\text{M}$ neostigmine before (a) or 30 min after a further treatment with $1.25 \mu\text{M}$ μ -conotoxin (b). The resting membrane potentials were -72 mV (a) and -74 mV (b). The contraction tracing (c) was taken from another preparation treated with neostigmine and μ -conotoxin. Phrenic nerves were stimulated with a train of pulse at 50 Hz for about 3 s (as indicated in the bottom). Calibrations: 1 s and 25 mV (a,b) or 0.5 g (c). Note that the muscle action potentials were observed in (a) but not in (b).

phenomenon. It may be inferred that the long-lasting contracture is caused by the intensive prolonged endplate depolarization, though the duration of the former greatly outlasts that of the latter. The mechanism that provokes the regenerative release of ACh and hence the prolonged endplate depolarization appears to involve activation of presynaptic ACh autoreceptors and Ca^{2+} channels (Hong & Chang, 1989a; Hong *et al.*, 1992). Since vesamicol inhibits ACh mobilization and enhances run-down of endplate current which are composed of quantal releases (Pemberton *et al.*, 1992), whether the mode of the unusual regenerative release of ACh is non-quantal (Zemková *et al.*, 1990) or quantal remains to be elucidated. The inhibitory effects of high Ca^{2+} Tyrode solution on the prolonged depolarization and tonic contracture may be due to activation of the large conductance Ca^{2+} -activated K^{+} channels (Anderson *et al.*, 1988a,b) or desensitization of nicotinic receptors (Giniatullin *et al.*, 1989). The results using long pulse stimulation also

demonstrate that a mechanism to provoke prolonged contracture upon prolonged depolarization exists along the whole muscle fibre. The mechanism appears not to be triggered by repetitive short pulses. The duration of the tonic contractures, whether induced by direct stimulation or by an indirect train of stimulation in the presence of neostigmine, appears to be related to the length of membrane depolarization. The dose-dependent shortenings of both phenomena by tubocurarine are in support of the idea that the tonic contracture is related to prolonged depolarization.

The tonic contracture is characterized by slow rise and slow fall of tension in comparison with the fast rise and decay of normal tetanic contraction. Moreover, the contracture lasted longer than the prolonged depolarization. It seems that, unlike the brief elevation of intracellular Ca^{2+} concentration triggered by muscle action potential, the prolonged depolarization might increase the cellular Ca^{2+} level continuously for an extended period of time. Since the Ca^{2+}

Figure 7 Contractions of mouse diaphragm evoked by direct stimulation. (a) The preparation was incubated in Ca^{2+} -free Tyrode solution containing EGTA (3 mM), tetrodotoxin (10 μM) and tubocurarine (10 μM) for 15 min and then stimulated with a pulse of 100 ms duration (I). (b) The preparation was pretreated with μ -conotoxin (1.25 μM) for 30 min and directly stimulated at 300 Hz for 3 s (pulse width 0.5 ms) before (b1) or 30 min after treatment with tetrodotoxin and tubocurarine (b2). Calibrations: 3 s and 1 g.

release from the sarcoplasmic reticulum is regulated by a Ca^{2+} -dependent inactivation process (Schneider & Simon, 1988), the present observations raise questions whether the prolonged depolarization provokes less inactivation process, desensitizes the inactivation mechanism or retards the Ca^{2+} uptake by sarcoplasmic reticulum.

Since the magnitude of the fullsize tonic contracture was about one tenth that of the maximal normal tetanic contraction, it may be assumed that about 10% of the contractile machinery is involved in the local contraction. This would mean that about 1 mm length of the muscle fibre of mouse diaphragm, the length of which is about 8–12 mm, partici-

pates in the contraction. This estimated length of muscle fibres is in accord with the length constant of the endplate depolarization but is more than 10 times larger than the surface dimension of the synaptic endplate (Salpeter & Eldefrawi, 1973; Daniello & Vogel, 1975). The observation that disturbances of the function of sarcoplasmic reticulum by formamide abolished the tonic contracture without impairment of generation of the prolonged endplate depolarization suggests that the majority of Ca^{2+} involved in the tonic contracture comes from the prolonged depolarization-induced Ca^{2+} release from subsynaptic sarcoplasmic reticulum, rather than from the Ca^{2+} influx through the ACh receptor ion channel. However, it cannot be ruled out that the mobilization of Ca^{2+} from intracellular stores by the ACh-dependent second messenger system (Giovannelli *et al.*, 1991) and the translocation of Ca^{2+} via the ACh receptor ion channel which becomes greater at more depolarized potentials (Decker & Dani, 1990) may contribute to some extent.

In mouse diaphragms treated with anticholinesterase, indirect stimulation produces a long-lasting Ca^{2+} signal at endplate region. This Ca^{2+} signal is different from the muscle action potential-induced fast Ca^{2+} transient in that it is slow in onset, enhanced by high Ca^{2+} solution and resistant to μ -conotoxin (Kimura *et al.*, 1991a,b). Although this slow Ca^{2+} signal was described as 'noncontractile', it is probable that the authors missed the low amplitude slow twitches (Figure 3c). Since under physiological conditions, e.p.p. itself produced no discernible local contraction it appears that the transient local depolarization of the endplate and Ca^{2+} influx across the postsynaptic membrane during the normal lifetime of transmitter action is not enough to cause contraction. On the other hand, prolonged and intensified activation of motor endplate nicotinic receptors by endogenous ACh can trigger a mechanism for prolonged local contractions. The possible profound increase in intracellular Ca^{2+} concentration at the nerve terminal and endplate region, as deduced from the intensive release of ACh and the tonic contracture, may bear a relation to the anticholinesterase-induced pathological changes of nerve endings and focal necrosis of muscle endplates (Laskowski *et al.*, 1975; Wecker *et al.*, 1978).

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