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THE NATURE AND ORIGIN OF CALCIUM-INSENSITIVE MINIATURE END-PLATE POTENTIALS AT RODENT NEUROMUSCULAR JUNCTIONS

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SUMMARY

1. To study the nature and origin of slow-rising, Ca^{2+}-insensitive miniature end-plate potentials (m.e.p.p.s) in mammalian muscle we used intracellular recording techniques and drugs which block acetylcholine (ACh) synthesis or the uptake of ACh into synaptic vesicles. Slow m.e.p.p.s were induced *in vivo* by paralysing the extensor digitorum longus muscle of the rat with botulinum toxin type A or *in vitro* by the application of 4-aminoquinoline to the mouse diaphragm nerve–muscle preparation.

2. Hemicholinium-3, which blocks ACh synthesis, reduced the amplitude of all synaptic potentials including slow m.e.p.p.s, but only if the nerve was stimulated.

3. 2(4-phenylpiperidino)cyclohexanol (AH-5183), which blocks the active uptake of ACh into synaptic vesicles, reduced both the frequency and the amplitude of slow m.e.p.p.s and did so without requiring nerve stimulation.

4. No correlation was observed between the molecular leakage of ACh from the motor nerve and the frequency and amplitude of slow m.e.p.p.s.

5. We conclude that slow m.e.p.p.s are caused by the release of ACh from the nerve terminal, possibly from a small pool of synaptic vesicle-like structures.

INTRODUCTION

Spontaneous release of acetylcholine (ACh) from motor nerve terminals, as initially demonstrated by Fatt & Katz (1952), gives rise to subthreshold synaptic potentials of uniform amplitude and time course called miniature end-plate potentials (m.e.p.p.s). The potentials are quantal in nature and are believed to originate from the transmitter content of a single synaptic vesicle. Another type of spontaneous transmitter release gives rise to a sustained depolarization of the end-plate region and is most probably caused by molecular leakage of ACh from the nerve terminal cytoplasm (Katz & Miledi, 1977; Vyskočil & Illés, 1977).

Recently, the appearance and properties of a third type of spontaneous transmitter release were reviewed (Thesleff & Molgó, 1983). The release is characterized by an

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unusual type of synaptic potential as first described by Liley (1957) in rat skeletal muscle. The potentials generally have a prolonged time-to-peak (1·5–10 ms) and varied amplitude from 0·1–15 mV (Kim, Lømo, Lupa & Thesleff, 1984). As discussed by Jansen & Van Essen (1976), they are probably not caused by a summation of m.e.p.p.s and are therefore not of the same quantal origin. The term slow m.e.p.p.s has been proposed for this type of potential (Kim *et al.* 1984). Slow m.e.p.p.s are particularly prominent in muscles paralysed by botulinum toxin type A (BoTx) as described previously (Colméus, Gomez, Molgó & Thesleff, 1982; Kim *et al.* 1984). The drug 4-aminoquinoline (4-AQ) has been shown to selectively enhance the frequency of slow m.e.p.p.s in both normal and BoTx-poisoned muscles (Molgó & Thesleff, 1982).

A major difference between m.e.p.p.s and slow m.e.p.p.s is manifested in their respective Ca^{2+} sensitivity. While the frequency of m.e.p.p.s is dependent upon the intraterminal Ca^{2+} concentration, the frequency of slow m.e.p.p.s is unaltered by procedures which elevate the Ca^{2+} concentration, e.g. by short-term nerve stimulation, nerve terminal depolarization and increases in the extracellular Ca^{2+} concentration (Liley, 1957; Thesleff, Molgó & Lundh, 1983). In this respect slow m.e.p.p.s behave similarly to the molecular leakage of ACh which also has been shown to be relatively resistant to K^+ and Ca^{2+} changes and to nerve stimulation (Vizi & Vyskočil, 1979; Katz & Miledi, 1981; Vyskočil, Nikolsky & Edwards, 1983).

To elucidate the nature and origin of slow m.e.p.p.s, we have studied the effects of drugs which interfere with certain specific steps in the synthesis, storage and release of ACh from nerve terminals. We have used hemicholinium-3 (HC-3), which interferes with the synthesis of ACh by blocking the high-affinity uptake of choline into nerve terminals (Hebb, Ling, McGeer, McGeer & Perkins, 1964; Guyenet, Lefresne, Rossier, Beaujouan & Glowinski, 1973). Another drug employed was 2(4-phenylpiperidino)cyclohexanol (AH-5183), which blocks the uptake of ACh into synaptic vesicles and also the molecular leakage of ACh (Marshall, 1970; Anderson, King & Parsons, 1983*a*; Edwards, Dolezal, Tuček, Zemkova & Vyskočil, 1985). Since this drug proved to be an efficient blocker of slow m.e.p.p.s several experiments were made to establish if a connexion existed between slow m.e.p.p.s and the molecular ACh leakage.

METHODS

The experiments were performed on the isolated extensor digitorum longus muscle (e.d.l.) of Sprague–Dawley rats (200 ± 20 g body weight) and the hemidiaphragm of the mouse (25 ± 5 g body weight). The muscles were removed from animals killed by diethylether (rats) or by cervical dislocation (mice).

Induction of slow m.e.p.p.s

Clostridium botulinum toxin type A (hemagglutinine + neurotoxin) in phosphate buffer solution (Ambache, 1949) was injected as a single dose of 0·25 ml, representing approximately 60 pg of the purified neurotoxin, into the anterolateral region of the right hind leg of the rat, superficial to the e.d.l. muscle. As shown by Kim *et al.* (1984), this type of toxin administration induces, within 7–10 days, the appearance of slow m.e.p.p.s at a frequency of 0·1–1 Hz. Slow m.e.p.p.s were induced *in vitro* by adding 4-AQ (100–200 μM) to the bathing solution. As described previously (Molgó & Thesleff, 1982; Molgó, Gomez, Polak & Thesleff, 1982) the drug selectively enhances the frequency of slow m.e.p.p.s within 10–15 min of its application to either normal untreated or BoTx-poisoned muscles.

Experimental conditions for m.e.p.p. recording

The excised muscles were pinned at their resting length through their tendons to a Sylgard plate, transferred to a temperature-controlled (±0·5 °C) 25 ml bath, in which they were suffused at the rate of 4–5 ml/min with aerated (95 % O_2 + 5 % CO_2) saline of the following composition (mM): NaCl, 135; KCl, 5; $CaCl_2$, 2; $MgCl_2$, 1; Na_2HPO_4, 1; $NaHCO_3$, 15; glucose, 11; pH, 7·2–7·3; temperature 30 °C, unless otherwise stated. In solutions with increased Ca^{2+}, iso-osmolarity was maintained by lowering of Na^+. In some experiments 2 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid (HEPES) buffer was used instead of $NaHCO_3$ and Na_2HPO_4. In experiments not involving nerve stimulation tetrodotoxin (TTX) at a concentration of 5×10^{-7} M was present in the medium to prevent action potentials from appearing in response to giant slow m.e.p.p.s.

Electrophysiological recordings. Potentials were recorded intracellularly by conventional glass capillary micro-electrodes filled with 2 M-K^+-citrate (resistances 5–8 × 10^6 Ω). Synaptic potentials were displayed on analogue and digital storage Tektronix D13 and 5223 oscilloscopes. The noise level of the recording circuit was less than 50 μV. surface and sometimes second layer muscle fibres were impaled with micro-electrodes in the end-plate area as localized visually with a dissection microscope at 100 × magnification. Focal recordings were considered those at which m.e.p.p.s with a time-to-peak of less than 0·8 and 1·1 ms were observed in preparations with active or inhibited cholinesterase, respectively. A bipolar platinum wire electrode was used for nerve stimulation (2–4 V; 50 μs).

Analysis of potentials. Synaptic potentials were analysed for frequency, amplitude and time-to-peak with an ABC 80 microcomputer with Data Disk 82 double floppy disk unit as described in detail by Colméus *et al.* (1982) and Kim *et al.* (1984). The amplitude of the potentials was calculated for a resting membrane potential of −75 mV and an ACh reversal potential of 0 mV (Katz & Thesleff, 1957). Unless otherwise stated, fibres with a resting membrane potential less than −55 mV were discarded. At each end-plate synaptic potentials were recorded during a period of 1–4 min. In each muscle six fibres were examined, as a minimum, during control and experimental conditions, analysed individually and expressed as means ± S.E. of means. Statistical significance was tested by means of Student's *t* test.

Leakage of ACh. The leakage of ACh was measured as mean hyperpolarization of membrane potential in the end-plate area in response to application of *d*-tubocurarine to muscles pre-treated with an anticholinesterase (Katz & Miledi, 1977; Vyskočil & Illés, 1977). These experiments were performed on the mouse hemidiaphragm preparation, carefully cleaned of pleura in the end-plate zone. The diaphragms were pre-treated with oxygenated saline solution containing 10^{-5} M-dietoxyparanitrophenylphosphate (Armin), an irreversible anticholinesterase, for 30 min at 20 °C, rinsed several times and then placed in the experimental chamber with oxygenated saline. The hemidiaphragm was about 1 mm below the surface of the solution which instead of being suffused was oxygenated by continuously blowing the gas mixture upon the surface (Vyskočil *et al.* 1983). Resting membrane potentials were measured from the oscilloscope screen, the measurements starting about 15 min after immersing the muscle in the bath. Recordings were made along the end-plate zone identified by the dissection microscope. Twenty-five or more fibres were routinely impaled before and 3–10 min after the addition of 10^{-5} M-*d*-tubocurarine to the solution (Vyskočil & Illés, 1978). Resting membrane potential measurements were also made in end-plate free areas of muscle fibres to ensure that the hyperpolarization was a local effect. At least three muscles were used for each experiment. The results are means from all recorded fibres ± S.E. of means.

Drugs and chemicals. 4-AQ, kindly supplied by Professor P. Lechat (Institut de Pharmacologie, Paris); 3,4-diaminopyridine (Aldrich, Belgium); diethoxyparanitrophenylphosphate (Armin), from the Institute of Physiology, Czechoslovak Academy of Sciences; *d*-tubocurarine (Burroughs & Wellcome Co., U.K.); HC-3, kindly supplied by Professor J. P. Long (University of Iowa, Iowa City, IA, U.S.A.); ouabain (Sigma, U.S.A.); AH-5183 (Pharmaceutical Research Department, Glaxo Group Research, U.K.); tetraphenylboron (Sigma, U.S.A.); TTX (Sankyo Co., Japan).

RESULTS

Effects of HC-3 on slow m.e.p.p.s

When HC-3 is added to a neuromuscular preparation it has no obvious effect on the amplitude of m.e.p.p.s. However, if the preparation is stimulated through its

nerve a gradual decrease occurs in m.e.p.p. amplitude (Thies, 1962; Elmqvist & Quastel, 1965). In this study we used this technique to see if HC-3 (5 μM) would similarly affect the amplitude of slow m.e.p.p.s and if such an effect would depend upon nerve stimulation. Fig. 1 illustrates m.e.p.p.s in untreated muscles and slow

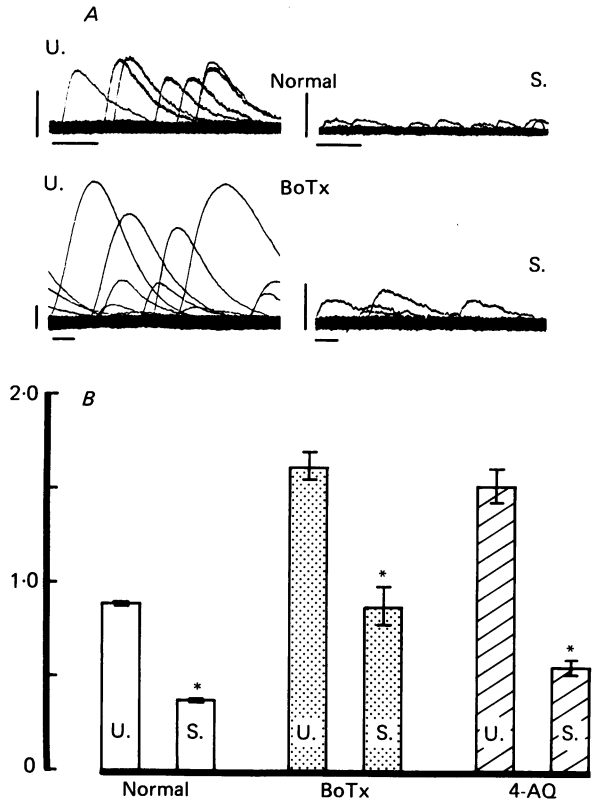

Fig. 1. Effect of nerve stimulation in the presence of HC-3 (5 μM) on the amplitude of m.e.p.p.s and slow m.e.p.p.s. U., unstimulated; S., stimulated. Stimulation rate was 10 Hz for 25 min. *A*, top row: oscilloscope tracings of m.e.p.p.s in a normal untreated mouse diaphragm in the presence of HC-3 without stimulation (left) and after 25 min of 10 Hz stimulation (right). Bottom row: oscilloscope tracings of m.e.p.p.s in a 14-day BoTx-poisoned rat e.d.l. muscle (left) and in another 14-day BoTx-poisoned e.d.l. muscle after 15 min of 10 Hz nerve stimulation in the presence of HC-3 (right). Calibrations are 1·0 mV and 2·0 ms for all traces. *B*, the effect of nerve stimulation in the presence of HC-3 on mean amplitude of m.e.p.p.s from normal untreated mouse diaphragm muscles, BoTx-poisoned rat e.d.l. muscles, and mouse diaphragms in the presence of 200 μM-4-AQ. Bars represent mean amplitude $\pm$ S.E. of mean. $*P < 0{\cdot}001$.

m.e.p.p.s induced by 4-AQ (200 μM) and by BoTx respectively in unstimulated muscles and following 25 min nerve stimulation at 10 Hz. In the case of BoTx-paralysed muscle 3,4-diaminopyridine (100 μM) was present to enhance evoked transmitter release during stimulation, but was not present during the recording of m.e.p.p.s. Note that the presence of slow m.e.p.p.s increases the mean amplitude of m.e.p.p.s recorded in the respective experiment. Stimulation in the presence of HC-3 greatly reduced the amplitude of all synaptic potentials as shown in the Figure, on average in experiments with m.e.p.p.s by 58 %, with slow m.e.p.p.s induced by 4-AQ

by 63 % and with BoTx by 46 %. Similar stimulation of the preparation in the absence of HC-3 caused no significant change in the frequency or amplitude of slow m.e.p.p.s. Fig. 1*A* shows typical examples of m.e.p.p.s in untreated and in BoTx-poisoned preparations with and without stimulation.

In the absence of stimulation HC-3 produced no reduction in m.e.p.p. amplitude within 140 min of observation, as shown in Fig. 2, indicating that at this concentration (5 μM) the drug had no noticeable post-synaptic receptor blocking activity.

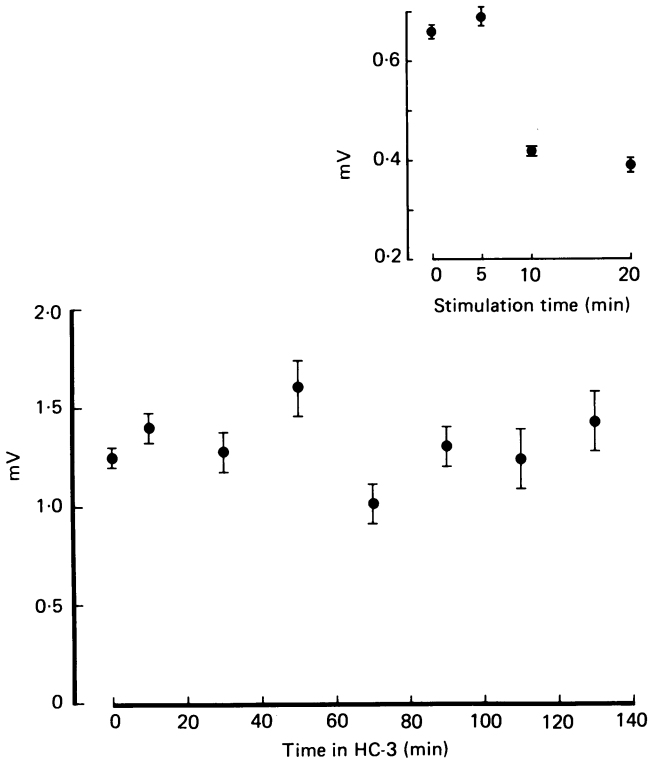

Fig. 2. Effect of HC-3 (5 μM) on the amplitude of m.e.p.p.s in resting conditions (main graph) and various times after nerve stimulation of 10 Hz (insert). All the values are means $\pm$ S.E. of means from two e.d.l. muscles 14 days after botulinum poisoning for the main graph and from a normal mouse diaphragm for the insert.

The insert in Fig. 2 shows that during stimulation there was no fall in the amplitude of m.e.p.p.s during the first 5 min of stimulation. Also, the reduction in m.e.p.p. amplitude recorded after stimulation in the presence of HC-3 was long-lasting and no recovery was seen within 40 min of observation. These results indicate that at this concentration HC-3 lacks 'open-channel blocking' activity and does not produce a use-dependent reduction in post-synaptic ACh sensitivity (Peper, Bradley & Dreyer, 1982; Gurney & Rang, 1984).

Effects of AH-5183 on slow m.e.p.p.s

AH-5183 is a drug which blocks the active uptake of ACh into synaptic vesicles (Anderson *et al.* 1983*a*). When applied to a normal neuromuscular preparation the

drug reduces the amplitude of evoked e.p.p.s and spontaneous m.e.p.p.s, but this effect appears only if transmitter release is stimulated (Van der Kloot, 1986).

The most prominent effect of AH-5183 (10^{-7} M) on slow m.e.p.p.s induced by BoTx was a reduction in frequency (Fig. 3), while increasing the concentration of AH-5183 10-fold (to 10^{-6} M) also reduced their amplitude as shown in Table 1. Slow m.e.p.p.s induced by 4-AQ were similarly reduced in amplitude by AH-5183, the reduction

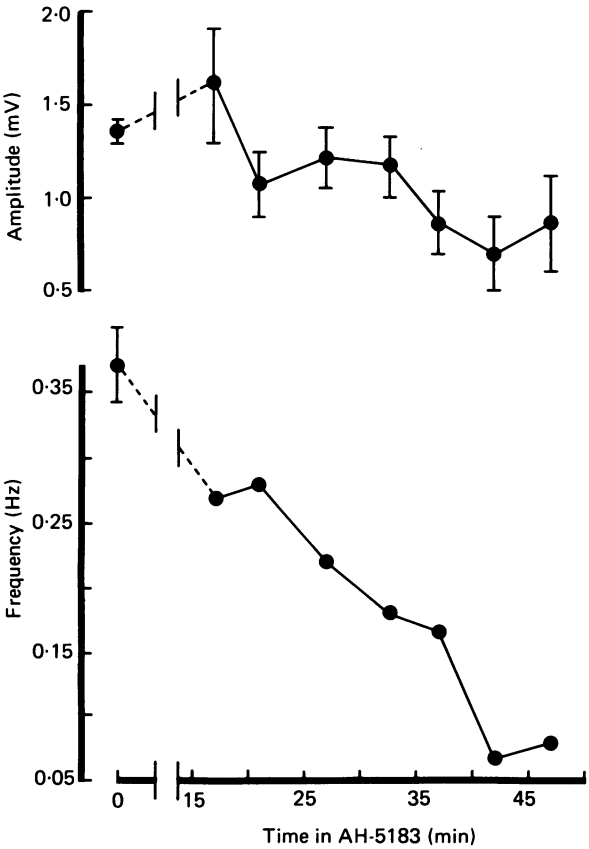

Fig. 3. The effect of 1 μM-AH-5183 on the amplitude and frequency of m.e.p.p.s from a rat e.d.l. muscle 14 days after botulinum poisoning. At time 0 the values are means $\pm$ S.E. of means from seven fibres recorded in normal Ringer solution. Starting at 15 min, the connected points represent values from a single fibre in the same muscle.

occurring within 15 min of application, but in this condition the drug (10^{-6} M) caused only a small reduction in frequency (Table 1). Similar results were obtained with tetraphenylborate (10^{-6} M) which is another drug known to block the active uptake of ACh into synaptic vesicles (Anderson, King & Parsons, 1983*b*).

Since AH-5183 has been shown not only to block the uptake of ACh into synaptic vesicles but also the leakage of ACh from the nerve terminal cytoplasm (Edwards *et al.* 1985, Vyskočil, 1985), the question arose which of these effects was responsible for the reduction in slow m.e.p.p. amplitude and frequency. Experiments were therefore made to see if a connexion existed between leakage of ACh and the slow m.e.p.p.s.

TABLE 1. Effects of AH-5183 (1 μM) on the frequency, amplitude and time-to-peak of m.e.p.p.s in control untreated mouse diaphragm muscles, mouse diaphragms bathed in 200 μM-4-AQ, and BoTx-poisoned (10–12 days) rat e.d.l. muscles. Values are expressed as means ± S.E. of means; figures in parentheses represent numbers of m.e.p.p.s/number of fibres/number of muscles, respectively, from which the results were calculated. *$P < 0.05$, **$P < 0.001$

	Frequency (Hz)	Amplitude (mV)	Time-to-peak (ms)
Control (621/11/2)	0·86 ± 0·17	1·1 ± 0·01	0·62 ± 0·02
AH-5183 (943/11/2)	1·57 ± 0·31	1·26 ± 0·002**	0·60 ± 0·01
4-AQ (831/8/2)	1·89 ± 0·21	1·23 ± 0·004	1·98 ± 0·006
AH-5183 (1051/15/2)	1·28 ± 0·14*	0·71 ± 0·02**	1·67 ± 0·04
BoTx (421/17/3)	0·27 ± 0·04	1·49 ± 0·06	2·48 ± 0·06
AH-5183 (76/13/3)	0·07 ± 0·01**	0·83 ± 0·09**	1·76 ± 0·09**

Comparison of leakage of ACh and slow m.e.p.p.s

The molecular leakage of ACh has been shown to depend on the activity of membrane-bound Mg^{2+}-dependent Na–K-ATPase, inhibition of the enzyme enhancing release while stimulation has an opposite effect (Vyskočil & Illés, 1977; Vizi & Vyskočil, 1979). Ouabain is a potent inhibitor of membrane ATPase (Skou, 1957) and has been shown to increase the leakage of ACh from cholinergic nerve terminals (Vizi, 1978; Vizi & Vyskočil, 1979). When tested on slow m.e.p.p.s in BoTx-poisoned muscles in a concentration of 2×10^{-4} M it was without significant effect on amplitude and frequency of slow m.e.p.p.s during 40 min of application.

Ca^{2+} in a concentration of 15 mM in the extracellular medium blocks the leakage of ACh as described by Vyskočil *et al.* (1983) and Sun & Poo (1985). The mean frequency of slow m.e.p.p.s in BoTx-poisoned muscles decreased slightly but insignificantly from 0·65 ± 0·12 Hz in 2 mM-Ca^{2+} to 0·50 ± 0·07 Hz in 15 mM-Ca^{2+}. This is quite unlike ACh leakage which is virtually abolished at this Ca^{2+} level. Neither did we observe changes in distribution of amplitude or time-to-peak of slow m.e.p.p.s in high Ca^{2+}. Values from ten fibres (322 m.e.p.p.s) in 2 mM-Ca^{2+} and ten other fibres (328 m.e.p.p.s) in 15 mM-Ca^{2+} respectively were as follows: amplitude 1·33 ± 0·42 and 1·19 ± 0·45 mV; time-to-peak 2·79 ± 0·55 and 2·89 ± 0·67 ms.

BoTx in a concentration corresponding to about 500 mouse LD_{50}/ml bathing solution has been shown to promptly inhibit leakage of ACh from motor nerve terminals (Dolezal, Vyskočil & Tuček, 1983; Vyskočil *et al.* 1983). The application of this amount of BoTx to a nerve–muscle preparation with slow m.e.p.p.s induced by BoTx paralysis failed to affect the frequency of slow m.e.p.p.s within the experimental period of 60 min. The frequency of slow m.e.p.p.s was 0·39 ± 0·04 before and 0·36 ± 0·06 Hz after BoTx application.

Fig. 4 illustrates the temperature dependence of the process responsible for ACh leakage in the mouse diaphragm between 10 and 30 °C. At 10 °C the amplitude of the hyperpolarization response at the end-plate was about 6 mV. It increased to over 12 mV at 20 °C which corresponds to a temperature coefficient of 2·35. The frequency of slow m.e.p.p.s rises much more quickly and increases more than 12 times between 10 and 22 °C (Thesleff *et al.* 1983). Thus, the two processes of ACh release possess quite different temperature dependences.

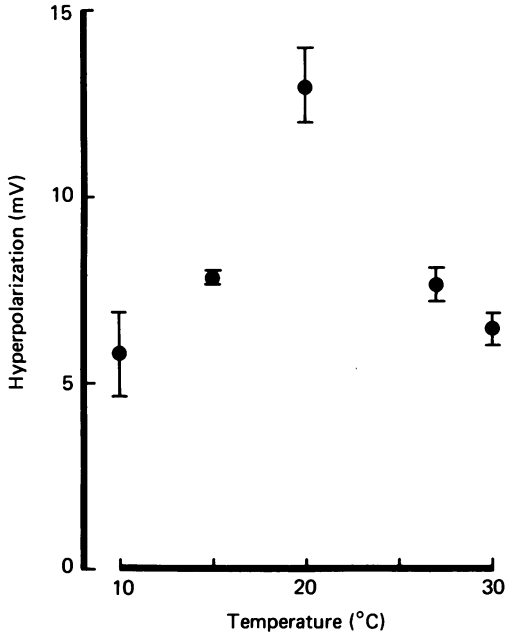

Fig. 4. Temperature dependence of the molecular ACh leakage at mouse diaphragm neuromuscular junctions. All muscles were pre-treated for 30 min with 10 μM-Armin. Each point represents mean $\pm$ S.E. of mean of the hyperpolarization induced at the end-plate region by *d*-tubocurarine in two to four muscles.

It is possible to activate the Na–K-ATPase and thereby to inhibit ACh leakage by placing the nerve–muscle preparation in cold (7 °C) over night and then to rewarm the preparation to 37 °C. In the cold the tissue accumulates Na^+ and upon rewarming the membrane Na–K-ATPase is activated and ACh leakage blocked (Kernan, 1962; Vizi &Vyskočil, 1979). when this procedure was carried out on BoTx-poisoned muscles it was observed that slow m.e.p.p.s appeared almost immediately upon rewarming the tissue, i.e. at a time when the ion pump was activated and ACh leakage stopped (Vizi & Vyskočil, 1979). As shown by Fig. 5 neither the frequency, time-to-peak nor amplitude of m.e.p.p.s in the presence of 4-AQ were significantly different from the potentials recorded in hemidiaphragm muscles not subjected to these temperature changes. Similar results were obtained in BoTx-poisoned rat e.d.l. muscles.

Consequently, it can be concluded that neither membrane Na–K-ATPase inhibition nor its activation has any effect on slow m.e.p.p.s present in BoTx- and 4-AQ-treated muscles.

The question arose if 4-AQ, which in acute experiments stimulates the mechanism responsible for slow m.e.p.p.s, might also affect the leakage of ACh. Table 2 demonstrates that the mean resting membrane potential of muscle fibres in the end-plate zone of anti-esterase-treated diaphragms was not changed 15–30 min after the application of 2×10^{-4} M-4-AQ, and that the hyperpolarization produced by *d*-tubocurarine was 6·5 mV in control and 6·2 mV in 4-AQ-treated specimens. The drug 4-AQ therefore possesses neither stimulatory nor blocking effects on the molecular leakage of ACh from the nerve terminal.

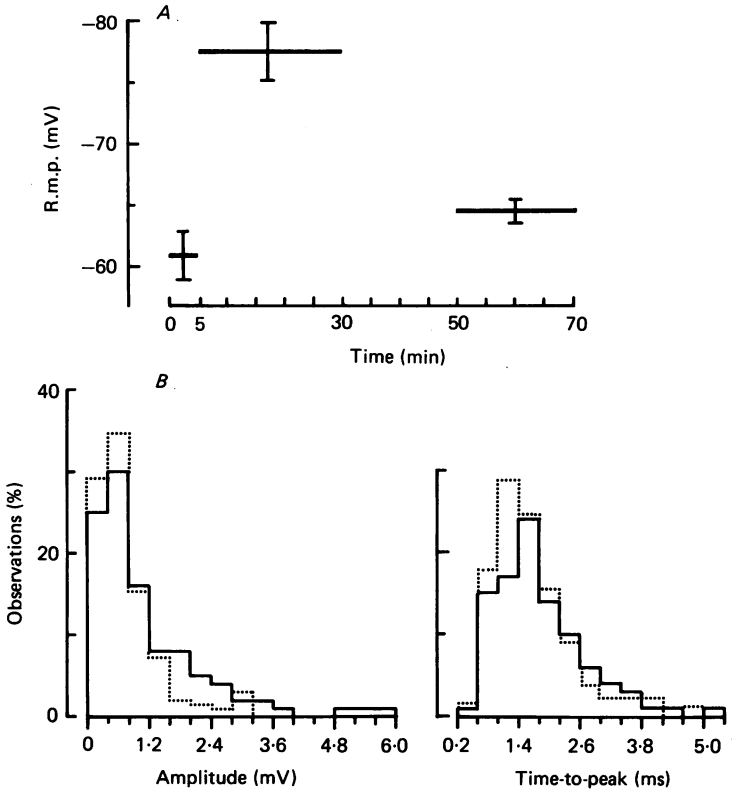

Fig. 5. Effect of Na pump activation on *A*, junctional resting membrane potential (r.m.p.) and *B*, amplitude and time-to-peak of m.e.p.p.s in a mouse diaphragm preparation in the presence of 200 μM-4-AQ. The muscle was kept for 13 h at 7 °C and then placed in the muscle bath at 30 °C at time = 0. *A*, mean values (±S.E. of mean) of junctional resting membrane potentials 0–5, 5–30, and 50–70 min after placing the muscle in 30 °C. Note the period of hyperpolarization from 5 to 30 min, indicating the activation of the Na–K-ATPase. *B*, amplitude and time-to-peak distribution histograms for m.e.p.p.s recorded 5–30 min (continuous line) and 50–70 min (dotted line) after placing the muscle in 30 °C. Mean m.e.p.p. frequency was 1·49±0·17 Hz at 5–30 min and 1·29±0·17 Hz from 50 to 70 min.

TABLE 2. Mean junctional resting membrane potentials (±S.E. of mean) in control untreated muscles and in the presence of 4-AQ before and after addition of 10 μM-*d*-tubocurarine. The muscles were pre-treated for 30 min with 10 μM-Armin (see Methods). Data from two experiments for each, number of fibres in parentheses. Temperature = 30 °C

	Normal Ringer solution	*d*-Tubocurarine	Hyperpolarization
Control muscle	−71·7±0·7 (37)	−78·2±0·9 (25)	6·5 mV
4-AQ 200 μM	−67·7±0·4 (41)	−73·9±0·9 (40)	6·2 mV

DISCUSSION

HC-3 reduced the amplitude of m.e.p.p.s and slow m.e.p.p.s only in stimulated preparations. Such an effect could be due to either a pre- or post-synaptic action of the drug. Since HC-3 failed to affect m.e.p.p. amplitude under resting conditions we

conclude that, at the concentration used, it lacks post-synaptic receptor blocking activity. The observations that it took several minutes of nerve stimulation for a reduction in m.e.p.p. amplitude to occur and that no recovery of amplitude was observed after the end of stimulation argues against the possibility that the drug exerted its effects through a use-dependent blockade of ACh channels. Since it is known that at the concentrations used HC-3 blocks the high affinity uptake of choline into nerve terminals we conclude that the observed reduction of m.e.p.p. amplitude is of presynaptic origin, involving a depletion of transmitter (Tagaki, Kojima, Nagata & Kuromi, 1970).

This conclusion provides further evidence that the agent responsible for slow m.e.p.p.s is ACh. Previous evidence supporting this idea are the findings that slow m.e.p.p.s are blocked by *d*-tubocurarine (Liley, 1957; Molgó & Thesleff, 1982) and enhanced in amplitude and time course by cholinesterase inhibition (Liley, 1957). The nerve-activity-dependent effect of HC-3 indicates that the ACh responsible for the slow m.e.p.p.s originates from the same store of transmitter as stimulus-evoked, Ca^{2+}-dependent release and that, consequently, ACh from the motor nerve terminal and not from the Schwann cell accounts for slow m.e.p.p.s.

Slow m.e.p.p.s were reduced in frequency and amplitude by the drug AH-5183, which blocks the active uptake of ACh from the cytoplasm into synaptic vesicles as well as the molecular leakage of ACh from the nerve terminal (Anderson *et al.* 1983*a*; Edwards *et al.* 1985; Vyskočil, 1985). Since it was possible to block or enhance the leakage of ACh by a variety of procedures which affected neither the frequency nor the amplitude of slow m.e.p.p.s we by exclusion suggest that the drug AH-5183 exerted its effects on slow m.e.p.p.s by blocking ACh transport into synaptic vesicles or similar intraterminal structures.

Normal m.e.p.p. amplitude is also reduced by AH-5183, but only if ACh release is increased by nerve stimulation or treatment with hypertonic Ringer solution (Van der Kloot, 1986). This is presumably because of the large number of synaptic vesicles present in the nerve terminal. In contrast, slow m.e.p.p.s were reduced in amplitude and frequency by AH-5183 without nerve stimulation, indicating that the number of vesicle-like structures responsible for slow m.e.p.p.s is comparatively small.

In conclusion, we interpret our results to suggest that slow m.e.p.p.s are caused by the spontaneous release of ACh synthesized within the nerve terminal cytoplasm. The release of ACh occurs from a small pool of membrane-lined structures with synaptic-vesicle-like properties, i.e. possibly from clusters or rows of synaptic vesicles which are located outside of the active zones and not affected by Ca^{2+}.

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