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THE EFFECT OF NON-QUANTAL ACETYLCHOLINE RELEASE ON QUANTAL MINIATURE CURRENTS AT MOUSE DIAPHRAGM

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SUMMARY

1. The amplitude and exponential decay time constant of miniature endplate currents (MEPCs) were measured in mouse diaphragms treated with anti-cholinesterase under conditions known to modulate non-quantal acetylcholine (ACh) release.

2. Anti-cholinesterase prolonged MEPC decay and the extent of this initial prolongation was not influenced by non-quantal release. When non-quantal release was present, the decays of MEPCs became increasingly faster over several hours. This increased decay did not occur in the absence of non-quantal release.

3. Potentiation of the non-quantal release by zero Mg^{2+} and 1×10^{-5} M choline, on the other hand, led to acceleration of MEPC shortening.

4. Increase of temperature from 15 to 26 °C and the presence of the desensitization-promoting drug proadifen (5×10^{-6} M) accelerated the rate of MEPC shortening.

5. These observations are consistent with increased receptor desensitization due to non-quantal release. Repetitive binding of ACh to postsynaptic receptors which prolongs the time course of MEPC in anti-cholinesterase-treated endplates leads to progressive desensitization in the presence of non-quantal release and to the subsequent shortening of the quantal responses.

INTRODUCTION

In addition to the well-established quantal release of acetylcholine (ACh) from the nerve terminal, there is also non-quantal release (NQR) (Katz & Miledi, 1977; Vyskočil & Illés, 1977, 1978). In nerve–muscle preparations treated with an anti-cholinesterase (anti-AChE) the ACh released non-quantally reaches the postsynaptic receptors and causes a small depolarization of the membrane potential at the endplate region of the muscle fibres. This depolarization may be identified by the hyperpolarization (H-effect) seen after the blockade of ACh receptors with (+)-tubocurarine. Under certain conditions, the ACh released in this manner can

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lead to a desensitization of the ACh receptors and can eventually prevent impulse transmission (Vyskočil, Nikolsky & Edwards, 1983). There is evidence that suggests that the quantal and non-quantal release influence each other. Specifically, increases in quantal release potentiate the NQR (Zemková, Vyskočil & Edwards, 1990; Nikolsy, Voronin, Oranska & Vyskočil, 1991). Here we examined the question of whether the amplitude and duration of quantal events can be modified by ACh released in a non-quantal manner. The kinetic parameters of quantal MEPCs change during manipulation of NQR, indicating direct interaction between transmitter from both types of release. This interaction is discussed in terms of the repetitive binding of ACh to receptors and desensitization.

METHODS

The experiments were performed *in vitro* on diaphragm strips dissected quickly from female mice (20–25 g body weight) killed by cervical dislocation. The muscles were placed into a 2 ml perfusion chamber with a Peltier cooling device and continuously superfused with Krebs–Ringer solution (mM: NaCl, 120; KCl, 5; $CaCl_2$, 2; $MgCl_2$, 1; NaH_2PO_4, 1; $NaHCO_3$, 24; glucose, 17; pH 7·2–7·4) continuously aerated with a 95% O_2 and 5% CO_2 mixture. The perfusion rate was 2 ml min^{-1}. Unless otherwise stated, experiments were performed at 20 °C. For intracellular recording of the MEPCs a standard two-microelectrode voltage-clamp technique was used and the membrane potential was held at −70 mV. The frequency band of the recording was 0–3000 Hz. At least 100 events from the bell-shaped part of the amplitude histogram were captured from each fibre and analysed by a computer for both amplitude and time constant of the exponential decay (τ_{MEPC}). Non-quantal ACh release was measured using the H-effect (Katz & Miledi, 1977; Vyskočil & Illés, 1977, 1978), i.e. the difference between the resting membrane potential recorded from the focal endplate zones of twenty-five muscle fibres before and of that 10 min after (+)-tubocurarine application (Vyskočil & Illés, 1978).

Acetylcholinesterase (AChE) was inhibited by 30 min pretreatment of the preparation with 1×10^{-5} M Armin (diethoxy-*p*-nitrophenylphosphate; Institute of Organic Chemistry, Academy of Science, Moscow, USSR), an irreversible organophosphate drug, or by continuous bathing with 3×10^{-6} M neostigmine (Sigma, USA).

Some diaphragms were denervated under ether anaesthesia using sterile precautions. The phrenic nerve was cut intrathoracically as described elsewhere (Beránek & Vyskočil, 1967).

The results were expressed as the mean of the indicated value obtained from all the fibres (n) ± S.E.M. The statistical significance of the differences was evaluated by Wilcoxon's non-parametric test at a probability level (P) of 0·05.

RESULTS

Amplitude and τ_{MEPC} of the MEPC during spontaneous decrease of NQR

The H-effect has been shown to decrease spontaneously with time (Nikolsky *et al.* 1991). MEPC amplitude, τ_{MEPC} and the amplitude of the H-effect were therefore followed for 3 h in six muscles starting 15–20 min after removal of the anti-AChE drug armin (Fig. 1). Initially, the H-effect, reflecting NQR, was 5·8 ± 0·5 mV ($n = 6$, time zero on Fig. 1). MEPC amplitude and τ_{MEPC} were 3·0 ± 0·5 nA and 5·4 ± 0·6 ms ($n = 10$).

The H-effect declined to zero within 3 h and the τ_{MEPC} decreased in parallel from 5·4 ± 0·2 to 3·1 ± 0·3 ms ($P > 0·05$, $n = 6$). There was no significant change in MEPC amplitude throughout the experiment. Similar results were obtained when the reversible anti-cholinesterase neostigmine (3×10^{-6} M) was present in the bath throughout the experiment (data not shown).

Measurements of τ_{MEPC} and MEPC amplitude in five diaphragms untreated with anti-AChE showed no substantial changes with time (Fig. 1). τ_{EPC} was $1{\cdot}4 \pm 0{\cdot}2$ ms at the beginning and remained stable over 4 h, as did the amplitude (not given).

Magnesium and MEPC

It has been shown that non-quantal release is inhibited by Mg^{2+} in the medium (Zemková & Vyskočil, 1989). It is high in Mg^{2+}-free saline and is almost zero at

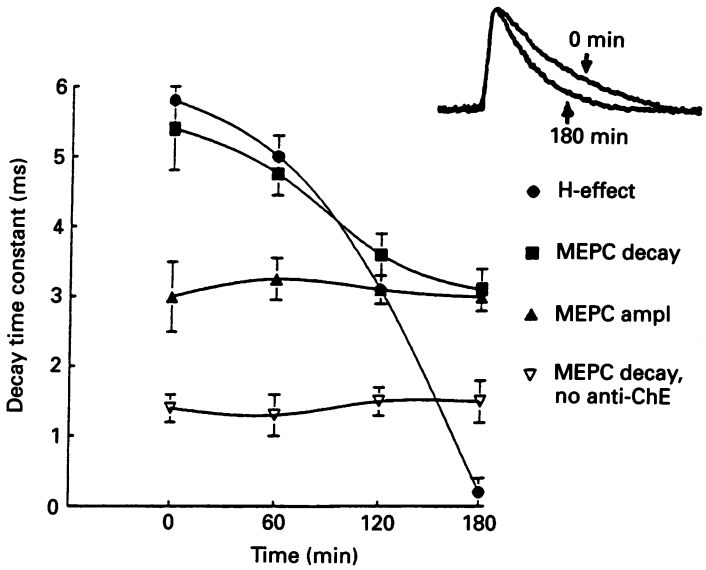

Fig. 1. The time course (abscissa in minutes) of changes in the decay time constant of miniature endplate currents (MEPC decay; ■) and their amplitude (nA, MEPC ampl; ▲) during spontaneous decrease of non-quantal acetylcholine release, expressed as the H-effect (mV, ●), in endplate treated with an anti-cholinesterase (anti-ChE) (ordinate). Open triangles show the decay time constant of MEPCs recorded from 6 control muscles untreated with anti-ChE. Otherwise, 6–10 muscles were used and in each 3-4 endplates were recorded at any given time (±2 min), as indicated. The inset shows the interposed mean time course of 100 MEPCs recorded and averaged from the same endplate at time zero (+4 min) and 180 min after anti-ChE treatment.

concentrations of 3 mM or higher. τ_{MEPC} and amplitude were therefore measured in 0, 1 (normal) and 3 mM Mg^{2+} saline (Fig. 2*A*).

There were only small changes in τ_{MEPC} when non-quantal release was eliminated by 3 mM Mg^{2+}. To check that non-quantal release was indeed absent, H-effect was assessed independently in several muscles at the end of MEPC measurements.

When magnesium was absent from the bathing solution, the decay was markedly shortened and τ_{MEPC} reached values of about 2 ms within 180 min. Variations in Mg^{2+} concentration affected neither amplitude nor time course of the MEPC in preparations not treated with anti-ChE (Zemková & Vyskočil, 1989). Control MEPC amplitude was $3{\cdot}9 \pm 0{\cdot}5$ nA and $\tau = 1{\cdot}43 \pm 0{\cdot}1$ ms ($n = 10$ fibres); with 3 mM Mg^{2+} it was $3{\cdot}7 \pm 0{\cdot}4$ nA and $1{\cdot}47 \pm 0{\cdot}2$ ms respectively ($n = 4$ fibres).

Choline and MEPC

The non-quantal release is more pronounced and prolonged with 1×10^{-5} M choline in the bath (Nikolsky *et al.* 1991). The time course of the change in MEPC shortening was faster in the presence of choline than in control choline-free medium (Fig. 2*B*).

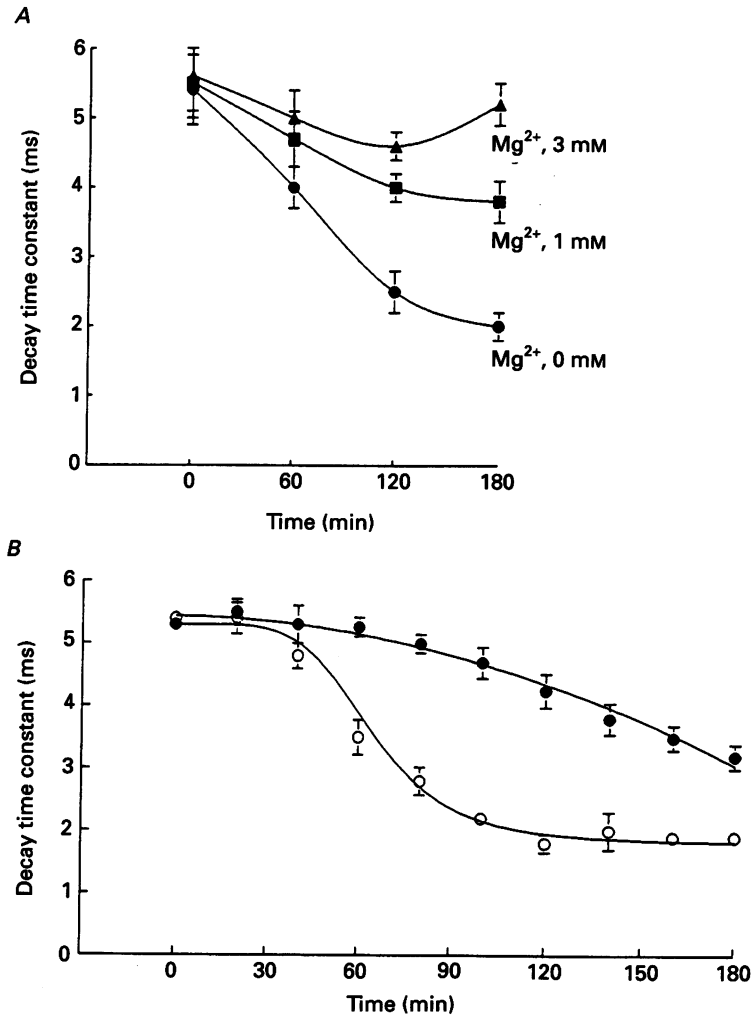

Fig. 2. *A*, the time course (abscissa in minutes) of changes in the decay time constant of the miniature endplate currents (ordinate in milliseconds) measured in anti-cholinesterase-treated diaphragms bathed with saline containing 0, 1 and 3 mM magnesium. Twenty-one endplates from 6 muscles were studied. *B*, the time course (abscissa in minutes) of MEPC decay time constant changes (ordinate in milliseconds) in anti-ChE-treated endplates bathed either in the control solution (●) or in the presence of 1×10^{-5} M choline (○). Each point represents a mean of measurements from 10–12 endplates.

Denervation and MEPC

It is known that in the diaphragm non-quantal release is decreased (rat, Zemková, Vyskočil & Edwards, 1987) or even absent (mouse, Nikolsky, Voronin & Oranska,

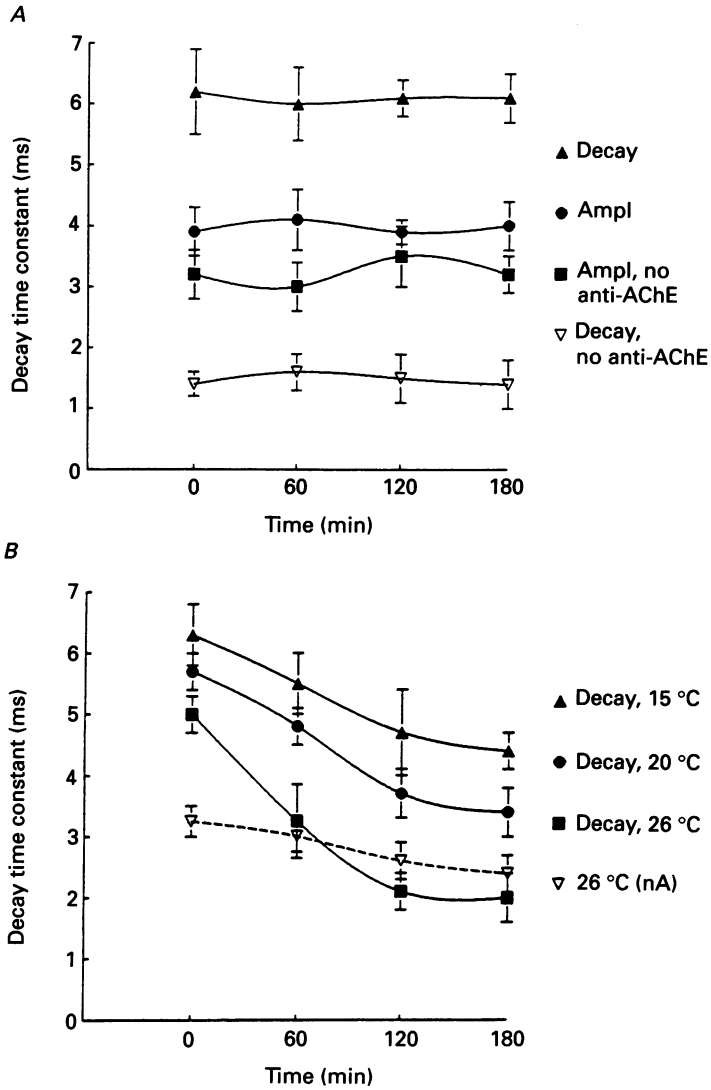

Fig. 3. *A*, the time course (abscissa in minutes) of the amplitude (nA, ampl) and decay time constant (ms, decay) of miniature endplate currents measured in anti-AChE-treated (▲, ●) and non-treated (■, ▽, no anti-AChE) diaphragms, denervated intrathoracically for 4 h. *B*, the time constant of MEPC decay time constant (ordinate in milliseconds) at three different temperatures. Open triangles are MEPC amplitude changes at 26 °C. Each point is a mean of measurements from 8–10 endplates.

1985) within 4 h of section of the phrenic nerve *in vivo*. At that time the quantal release of ACh is maintained and MEPC frequency and other parameters are unchanged. In 4-h-denervated muscles with intact AChE, the MEPC amplitude was $3{\cdot}2 \pm 0{\cdot}4$ nA and $\tau_{MEPC} = 1{\cdot}4 \pm 0{\cdot}2$ ms ($n = 8$). These values are similar to those obtained from innervated endplates (amplitude $= 2{\cdot}9 \pm 0{\cdot}5$, $\tau_{MEPC} = 1{\cdot}5 \pm 0{\cdot}3$ ms, $n = 8$).

When the denervated muscles were bathed with Armin, their MEPCs increased in amplitude to $3{\cdot}9 \pm 0{\cdot}8$ nA and τ_{MEPC} to $6{\cdot}2 \pm 0{\cdot}7$ ms ($n = 9$) (similar values to those recorded in innervated diaphragms treated with Armin). However, in contrast to innervated muscles (see Fig. 1), there was no shortening of the time constant of decay

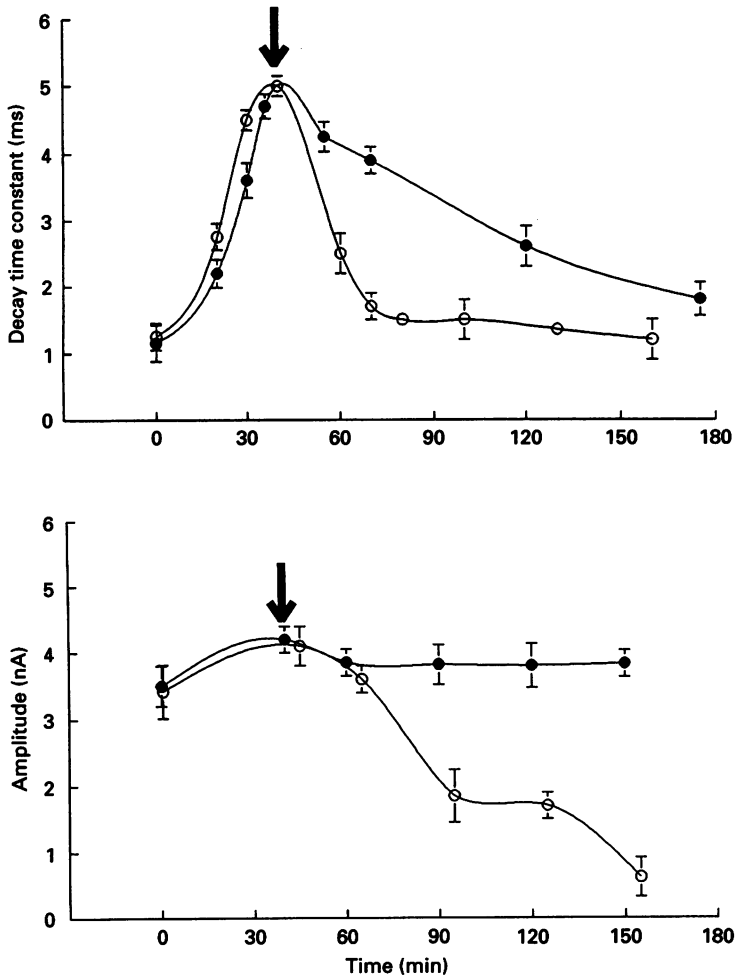

Fig. 4. The time course (abscissa in minutes) of changes in MEPC decay time constant (upper trace, ordinate in milliseconds) and amplitude (lower part, ordinate in nanoamperes) in control (●) and proadifen-treated muscles (5×10^{-6} M, arrow, ○). The figure also shows the initial increase of MEPC decay and amplitude after application of 3×10^{-6} M neostigmine (at time zero) into the muscle bath. Mean values from 10–12 endplates (4 muscles).

during the next 3 h (Fig. 3*A*). Tests at various times showed no H-effect throughout this period. This suggests that the reduced time of decay observed in innervated muscles is related to the presence of non-quantal release and may well be due to desensitization developing as a result of the ACh released both quantally and non-quantally.

Temperature, proadifen and MEPC

It is known that the time course of desensitization is accelerated at higher temperatures and is slowed by cooling (Magazanik & Vyskočil, 1975). Amplitude and τ_{MEPC} were therefore estimated at 15, 20 and 26 °C. After anti-AChE treatment at 26 °C there was a marked prolongation of MEPC decay within the first few minutes, and τ_{MEPC} was 6·9 times that before anti-AChE, whereas at 20 °C there was only a 3·1-fold difference. The time course of the reduction of τ_{MEPC} was slowest at 15 °C and fastest at 26 °C (Fig. 3*B*) in agreement with the postulated role of desensitization. At 26 °C, a significant reduction in MEPC amplitude was observed, another indication that desensitization had occurred. This decrease in amplitude was absent in muscles with intact AChE at 26 °C (data not given).

Proadifen (5×10^{-6} M), is known to potentiate the desensitization of the ACh receptor (Magazanik & Vyskočil, 1973; Giniatullin *et al.* 1989). Addition of proadifen to the bath (20 °C) initially accelerated the shortening of MEPC in preparations treated with anti-AChE (Fig. 4). Shortly after this, MEPC amplitude began to decline, and was reduced to 15–20% at 120 min after addition of proadifen (cf. Giniatullin *et al.* 1989). This means that desensitization is manifested first as a shortening of MEPC decay time and thereafter as a drop in MEPC amplitude. Proadifen had no effect on endplates with intact AChE (Magazanik, Nikolsky & Vyskočil, 1982).

The phenomena described here was also seen in muscles bathed continuously with the reversible anti-cholinesterase neostigmine (3×10^{-6} M; Fig. 4). This eliminates the possibility of spontaneous reactivation of Armin-inhibited AChE.

DISCUSSION

We have shown that the prolongation of quantal MEPC decay induced by blocking AChE is not potentiated by non-quantal ACh release (cf. Figs 1 and 2, time zero, with Fig. 3). However, the subsequent shortening of the decay depends on non-quantal release as the MEPCs did not shorten in the absence of non-quantal release.

In anti-AChE-treated endplates the prolongation of the decay phase of a single quantal response (miniature endplate potential or MEPC) known as postsynaptic potentiation (Hartzel, Kuffler & Yoshikami, 1975; Feltz & Trautmann, 1980) is apparently a consequence of the repetitive binding and activation of postsynaptic receptors. The repetitive binding and the resulting prolongation of decay would be greater if both the concentration of ACh and receptor density were high. Conversely, repetitive binding and therefore decay time would be reduced if either the ACh concentration or the receptor density decreased, as happens in the presence of ACh receptor inhibitors or during desensitization. Proportional increase of repetitive binding and decay prolongation with higher ACh concentration should be observed only if two ACh molecules were required to activate one receptor (Colquhoun & Hawkes, 1981; Conti-Tronconti & Raftery, 1982). Otherwise, in the case of 1:1 stoichiometry of receptor activation, the increase in ACh concentration would decrease repetitive binding as a result of reduced availability of free receptors.

The concentration of ACh giving rise to the MEPC can be increased (without quantum size change) by the non-quantally released ACh which is present in the cleft (Vyskočil *et al.* 1983). However, there is no reduction of τ_{MEPC} when non-quantal release was eliminated suggesting that the non-quantal ACh does not enhance repetitive binding during quantal release. On the other hand, non-quantal ACh did produce a shortening of the quantal responses, most probably by desensitization. We propose that desensitization has in fact two stages: (a) shortening the responses when the decay is controlled to a substantial extent by repetitive ACh binding and when the initial number of receptors has not been markedly reduced either by pre-existing desensitization or by antagonists, and (b) a reduction in amplitude during the massive and more prolonged maintenance of the receptors in the desensitized state. Desensitization could shorten the responses in two ways: (i) by decreasing the density (number) of functioning receptors or (ii) by decreasing the amount of ACh available for repetitive binding by 'trapping' a portion of ACh molecules on inactive desensitized receptors. This 'trapping' could be quite significant since the affinity of desensitized receptors for ACh is increased by two orders of magnitude (Heidmann & Changeux, 1979; Cohen & Strnad, 1987; Magazanik, Snetkov, Giniatullin & Khazipov, 1990). Immediately after release, the quantal ACh activates the available receptors facing the active zone so that the MEPC amplitude is maximal (as indicated by the small amplitude increase after AChE inhibition, Fig. 4, lower trace). Amplitude remains almost maximal even if only a small fraction (say 5%) of the receptors are already desensitized. As a result of this initial activation, a further proportion of the receptors becomes desensitized, the number of receptors available for repetitive binding is decreased and the decay time is reduced. These desensitized receptors could continue to trap ACh molecules, thereby reducing even further the probability of repetitive binding. Therefore the initial effect of the desensitization-potentiating drug proadifen was not a decrease in amplitude but a shortening of the decay time (open circles in Fig. 4; cf. also Giniatullin *et al.* 1989). Non-quantal release might well maintain a certain number of ACh receptors in a desensitized or easily desensitizable state (Magleby & Pallota, 1981). This number depends on the extent of non-quantal release and on factors that increase the rate of desensitization.

Non-quantal release could also 'saturate' desensitized receptors and thus allow for quantally released ACh molecules to bind repetitively to non-desensitized receptors. After NQR cessation, ACh is 'trapped' on the desensitized receptors, so that the possibility of repetitive binding is reduced and MEPC decay is shorter.

This speculation on the role of non-quantal release and desensitization in shortening of the MEPC is supported by the present results. MEPC shortening was observed in all cases when non-quantal release was present. The time course of MEPC shortening was faster when NQR was greater (zero Mg^{2+}, choline present) and also when desensitization was increased at a higher temperature. At 26 °C, desensitization was apparently sufficient to diminish the number of functional receptors so that the amplitude of the MEPC declined. In this instance desensitization caused the shortening, not only by trapping ACh molecules, but also by significantly lowering the number of ACh receptors available for repetitive binding.

Again, no shortening was observed with 3 mM Mg^{2+}, after denervation, and at

15 °C. Under these conditions NQR was insignificant and in the latter case the desensitization rate was low.

Once developed, desensitization can last for minutes or longer (Magleby & Pallota, 1981; Magazanik *et al.* 1990). This in turn could explain the observation that the decay time was shortened even after cessation of NQR under normal conditions (Fig. 1). The proposed role of desensitization in the shortening of the quantal responses due to NQR is in agreement with earlier observations demonstrating a decrease in MEPC decay after application of the desensitization-potentiating drug proadifen to anti-AChE-treated frog neuromuscular junctions (Giniatullin *et al.* 1989).

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REFERENCES

BERÁNEK, R. & VYSKOČIL, F. (1967). The action of tubocurarine and atropine on the normal and denervated rat diaphragm. *Journal of Physiology* **188**, 53–66.

COHEN, J. B. & STRNAD, N. P. (1987). Permeability control and desensitization by nicotinic acetylcholine receptors. In *Molecular Mechanisms of Desensitization to Signal Molecules*, ed. KONJIN, T. M., VAN DE STARRE, H., VAN DER WEL, H. & HOUSLAY, M. D., pp. 257–273. Springer-Verlag, Berlin, Heidelberg.

COLQUHOUN, D. & HAWKES, A. G. (1981). On the stochastic properties of single ion channels. *Proceedings of the Royal Society* B **211**, 205–235.

CONTI-TRONCONTI, B. M. & RAFTERY, M. A. (1982). The nicotinic cholinergic receptor: correlation of molecular structure with functional properties. *Annual Review of Biochemistry* **51**, 491–530.

FELT, A. & TRAUTMANN, A. (1980). Interaction between nerve-released acetylcholine and bath applied agonists at the frog end-plate. *Journal of Physiology* **299**, 533–552.

GINIATULLIN, R. A., KHAMITOV, G., KHAZIPOV, R., MAGAZANIK, L. G., NIKOLSKY, E. E., SNETKOV, V. A. & VYSKOČIL, F. (1989). Development of desensitization during repetitive end-plate activity and single end-plate currents in frog muscle. *Journal of Physiology* **412**, 113–122.

HARTZELL, H. C., KUFFLER, S. W. & YOSHIKAMI, D. (1975). Postsynaptic potentiation: interaction between quanta of acetylcholine at the skeletal neuromuscular synapse. *Journal of Physiology* **251**, 427–463.

HEIDMANN, T. & CHANGEUX, J.-P. (1979). Fast kinetic studies on the interaction of a fluorescent agonist with the membrane-bound acetylcholine receptor from *Torpedo marmorata*. *European Journal of Biochemistry* **94**, 255–279.

KATZ, B. & MILEDI, R. (1977). Transmitter leakage from motor nerve endings. *Proceedings of the Royal Society* B **196**, 59–72.

MAGAZANIK, L. G., NIKOLSKY, E. E. & VYSKOČIL, F. (1982). Effect of the desensitization-potentiating agent SKF-525A on frog end-plate currents. *European Journal of Pharmacology* **80**, 115–119.

MAGAZANIK, L. G., SNETKOV, V. A., GINIATULLIN, R. V. & KHAZIPOV, R. N. (1990). Changes in the time course of miniature endplate currents induced by bath-applied acetylcholine. *Neuroscience Letters* **113**, 281–285.

MAGAZANIK, L. G. & VYSKOČIL, F. (1973). Desensitization at the motor endplate. In *Drug Receptors*, ed. RANG, H. P., pp. 105–119. Macmillan, London.

MAGAZANIK, L. G. & VYSKOČIL, F. (1975). The effect of temperature on desensitization kinetics at the postsynaptic membrane of the frog muscle fibre. *Journal of Physiology* **249**, 285–300.

MAGLEBY, K. L. & PALLOTTA, B. S. (1981). A study of desensitization of acetylcholine receptors using nerve-released transmitter in the frog. *Journal of Physiology* **316**, 255–250.

NIKOLSKY, E. E., VORONIN, V. A. & ORANSKA, T. I. (1985). Recovery of spontaneous quantal and non-quantal secretion of transmitter from motor nerve endings during mouse diaphragm reinnervation. *Doklady Akademii Nauk USSR* **285**, 246–249 (in Russian).

NIKOLSKY, E. E., VORONIN, V. A., ORANSKA, T. I. & VYSKOČIL, F. (1991). The dependence of non-quantal acetylcholine release on the choline-uptake system in the mouse diaphragm. *Pflügers Archiv* **418**, 74–78.

VYSKOČIL, F. & ILLÉS, P. (1977). Non-quantal release of transmitter at mouse neuromuscular junction and its dependence on the activity of Na^+,K^+-ATPase. *Pflügers Archiv* **370**, 295–297.

VYSKOČIL, F. & ILLÉS, P. (1978). Electrophysiological examination of transmitter release in non-quantal form in the mouse diaphragm and the activity of membrane ATPase. *Physiologia Bohemoslovaca* **27**, 449–455.

VYSKOČIL, F., NIKOLSKY, E. E. & EDWARDS, C. (1983). An analysis of the mechanisms controlling and underlying the non-quantal release of acetylcholine at the mouse neuromuscular junction. *Neuroscience* **9**, 429–435.

ZEMKOVÁ, H. & VYSKOČIL, F. (1989). Effects of Mg^{2+} on non-quantal acetylcholine release at the mouse neuromuscular junction. *Neuroscience Letters* **103**, 293–297.

ZEMKOVÁ, H., VYSKOČIL, F. & EDWARDS, C. (1987). A study on early post-denervation changes of non-quantal acetylcholine release in the rat diaphragm. *Pflügers Archiv* **409**, 540–546.

ZEMKOVÁ, H., VYSKOČIL, F. & EDWARDS, C. (1990). The effects of nerve terminal activity on non-quantal release of acetylcholine at the same neuromuscular junction. *Journal of Physiology* **423**, 631–640.