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THE EFFECTS OF NERVE TERMINAL ACTIVITY ON NON-QUANTAL RELEASE OF ACETYLCHOLINE AT THE MOUSE NEUROMUSCULAR JUNCTION

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SUMMARY

1. Local endplate depolarization induced by anticholinesterase application to mouse nerve–diaphragm preparations was taken as a measure of non-quantal release of acetylcholine.

2. Non-quantal acetylcholine release occurred within 20–60 s after anticholinesterase application, either spontaneously or evoked by nerve stimulation. Non-quantal release declined with time and disappeared after 3–5 min.

3. The amplitude of stimulation-evoked non-quantal release increased with the frequency of stimulation and was maximal at frequencies above 50 Hz. Two stimuli were sufficient to evoke the maximal effect.

4. Micromolar concentrations of atropine, pirenzepine and vesamicol reduced the amplitude and shortened the duration of non-quantal release. Oxotremorine (10^{-8} M) enhanced the amplitude and ouabain (10^{-4} M) prolonged the duration of non-quantal release.

5. Our results support the idea that the non-quantal release is due to the vesicular acetylcholine transport system which becomes transiently a part of the nerve terminal during exocytotic release of quantal acetylcholine.

INTRODUCTION

Non-quantal release of acetylcholine (ACh) from the nerve terminal at the murine neuromuscular junction has been attributed to the presence in the terminal membrane of a vesicular membrane ACh transport system which appears there as a consequence of the exocytic release of vesicular contents (Edwards, Doležal, Tuček, Zemková & Vyskočil, 1985; Vyskočil, 1985). Non-quantal release is usually measured soon after the addition of an anticholinesterase (e.g. paraoxon) to the bath. We have observed that the addition of an irreversible anticholinesterase causes extensive twitching and contractures, particularly in the region around the nerve terminal, as was described for prostigmine by Masland & Wigton (1940). Recently, we have found that the magnitude of the non-quantal release, as measured by the difference in

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membrane potential between the junctional and extrajunctional regions of anticholinesterase (anti-ChE)-treated muscle, decreases with time after wash-out of the anti-ChE and disappears within 1 h; the release can be restored by stimulating the motor nerve at 50 Hz for 1 min (Edwards, Doležal, Tuček, Zemková & Vyskočil, 1988). Therefore, it seemed likely that the explanation for the time course of the change of non-quantal release after wash-out of the anti-ChE is that the burst of activity following its addition produces massive exocytosis and therefore places a large number of ACh transporters in the nerve terminal membrane. The activity soon wanes, and there is a selective endocytosis of the transporters; thus the magnitude of the non-quantal release decrements with time. To support this proposed explanation, the effects on non-quantal release of stimulation and of agents which affect quantal and non-quantal release have been studied.

METHODS

Experiments were performed on the diaphragm muscles of female white mice of 20 ± 5 g body weight. After isolation, muscles were immersed in an oxygenated solution containing (in mM): NaCl, 137; KCl, 5·0; $CaCl_2$, 2·0; $MgCl_2$, 1·0; $NaHCO_3$, 12·0; NaH_2PO_4, 1·0; glucose, 11·0; pH 7·2 (Liley, 1956) and then treated with an irreversible anti-ChE (paraoxon, $2{\cdot}9 \times 10^{-5}$ M) for 15 min. All experiments were done at room temperature (20–24 °C).

The non-quantal release was determined as a local depolarization at the endplate zone of paraoxon-treated muscles (Katz & Miledi, 1977; Vyskočil & Illés, 1977) recorded extracellularly (Fatt, 1950). Strips of diaphragm muscles with the nerve were mounted vertically onto a glass rod frame $1{\cdot}5 \times 2{\cdot}5$ cm immersed in a chamber containing 5 ml of oxygenated solution. The upper and lower parts of the glass frame were coated with polyethylene tubing and the lateral parts of the frame were covered with petroleum jelly. The reference Ag–AgCl electrode was fixed on the top part of the frame just beneath the muscle strip. The recording Ag–AgCl electrode was placed in the bath (Fig. 1, inset). The potential differences between the electrodes were recorded during the repeated movement of the muscle up and down at a frequency of about one immersion every 2 s, displayed on a Tektronix oscilloscope and stored with a pen recorder. The potential differences along the muscle fibres changed transiently as the surface of the bath solution crossed the endplate zone: in control preparations, positive transients were seen regularly which corresponded to the hyperpolarization observed by intracellular microelectrode measurements (Vyskočil, 1974; Vyskočil & Illés, 1978; Vyskočil, Nikolsky & Edwards, 1983). The negative transients present at the endplate zone after cholinesterase inhibition corresponded to the local depolarization of the muscle fibre beneath the nerve ending previously measured with microelectrodes and in this paper it was assumed to measure the non-quantal release of ACh (Zemková & Vyskočil, 1989).

For nerve stimulation, platinum wire electrodes were fixed on the top of the chamber wall and the phrenic nerve was hung over them. The nerves were stimulated with 50 μs pulses of 5–10 V. In some experiments the presynaptic nerve terminal was depolarized directly by platinum electrodes placed at a distance of about 2 mm below and above the endplate zone.

Drugs and chemicals

Paraoxon (diethyl-*p*-nitrophenyl phosphate, Sigma) was applied from stock solution of $5{\cdot}8 \times 10^{-3}$ M. The drug vesamicol (2-(4-phenylpiperidino)cyclohexanol, AH 5183) was dissolved in 10% ethanol to give a stock solution of 10^{-4} M. Ouabain (Serva), *d*-tubocurarine (Burroughs–Wellcome), atropine (British Drug Houses, Ltd), pirenzepine, tetrodotoxin and oxotremorine (Sigma) were used. Marcaine (bupivacain hydrochloride) was provided by Astra (Switzerland). All chemicals for the physiological solutions were of Analar grade.

RESULTS

Spontaneous occurrence of non-quantal release

Within about 20–60 s after application of the irreversible anti-ChE to the freshly isolated (10–20 min) muscle, non-quantal release was found to be present, as shown

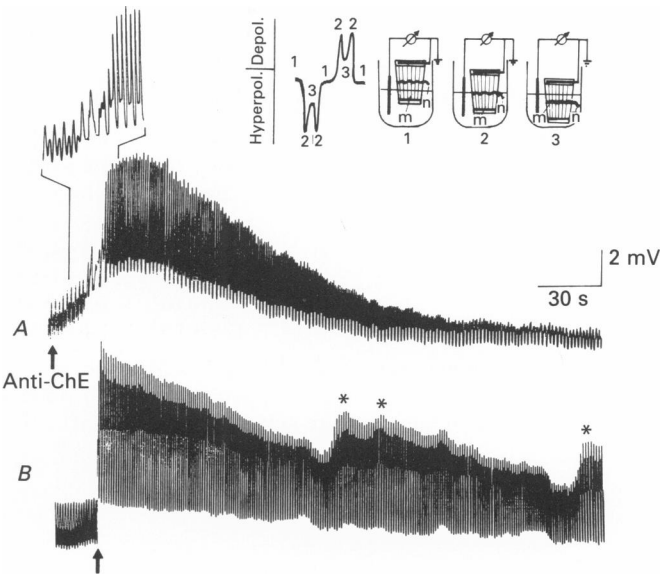

Fig. 1. Time course of the effects of application of anti-ChE to a freshly isolated muscle (*A*) or of stimulation (arrow) with two pulses at 100 Hz of a 2 h old preparation 60 s after anti-ChE application (*B*) on the endplate zone potential as recorded extracellularly. The records were obtained during repeated immersions of the muscles into the bath. The inset shows a record of the endplate hyperpolarization and depolarization responses and the corresponding positions of the muscle (m) during one cycle of immersion. In position 1, when the endplate zone (indicated by a nerve n, but actually it was localized around the smaller nerve branches above the main nerve trunk) was above the surface of the bath, the potential difference between recording electrodes was assumed to be zero. Position 2 represented the time when the endplate zone crossed the surface of the bath when a transient hyperpolarization (in control muscles) or depolarization (in anti-ChE-treated muscles) occurred. In position 3, when the endplate zone was below the bath surface, the potential should theoretically equal that in position 1 but this was not always so. The endplates were apparently asymmetrically distributed around the main endplate zone so that there were more endplates near the ribs (the top of the muscle) than near the tendon. Therefore, a small hyperpolarization (in control) or a small depolarization (after anti-ChE) persisted in the third position. The baseline shifts are due to muscle twitching. Asterisks indicate spontaneous reappearance of endplate depolarization.

by the rapid change of the extracellularly recorded local hyperpolarization to a depolarization (Fig. 1*A*). The magnitude of the non-quantal release reached a maximum and then decreased with time: it was half-maximal after about 70 s. In some muscles (which did not twitch) it was no longer measurable after 3–5 min. However, in most preparations spontaneous contractions were observed during (15 min) and occasionally after (10–60 min) anti-ChE application, and the non-quantal release reappeared spontaneously and then declined moderately (Fig. 1*B*).

TABLE 1. Maximal amplitude and half-decay time ($t_{\frac{1}{2}}$) of extracellularly recorded total endplate depolarizations evoked by nerve stimulation or by direct stimulation of the endplate zone (in the presence of tetrodotoxin and marcaine) with two pulses at 100 Hz after anti-ChE wash-out

		$t_{\frac{1}{2}}$ (s)	Amplitude (mV)	n
Control		70±5	3·3±0·3	15
Tetrodotoxin	10^{-8} M	65±7	3·2±0·4	4
Marcaine	10^{-5} M	60±10	3·0±0·6	4
Ouabain	10^{-4} M	132±22*	3·5±0·5	4
Atropine	10^{-7} M	45±10	2·0±0·5	4
	10^{-6} M	24±6*	1·2±0·3*	6
	10^{-6} M + Ouabain 10^{-4} M	168±18*	1·9±0·3*	6
Pirenzepine	10^{-7} M	20±7*	1·6±0·5*	5
	10^{-6} M	15±14*	1·5±0·4*	4
	10^{-7} M + Ouabain 10^{-4} M	160±7*	1·3±0·5*	4
Oxotremorine	10^{-8} M	52±13	5·5±0·8*	6
Vesamicol	10^{-7} M	25±10*	1·5±0·4*	4
	10^{-7} M + Ouabain 10^{-4} M	138±25*	1·4±0·5*	4

The drugs in the concentrations given were applied 10 min before and were present during the measurements. Values are given as mean±S.E.M. of n recordings. The differences between the control value and values after drug application are significant at $P < 0·01$ (*).

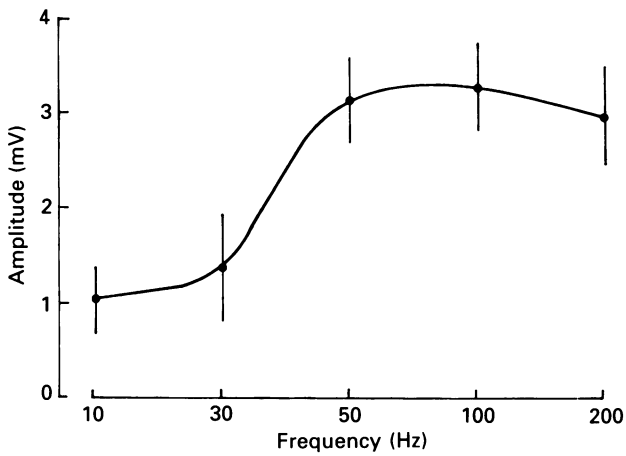

Fig. 2. Effect of stimulation frequency on maximal amplitude of non-quantal release in anti-ChE-treated preparations 10–60 min after wash-out. The nerve was stimulated with ten pulses. Values are given as a mean±S.E.M. of three to five recordings.

Block of the action potentials of the nerve fibres by the addition of tetrodotoxin (10^{-8} M) or the local anaesthetic marcaine (10^{-5} M) abolished the non-quantal release produced spontaneously by application of anti-ChE; however, in these preparations, the non-quantal release was easily evoked by direct stimulation of the endplate zone of the muscle (Table 1).

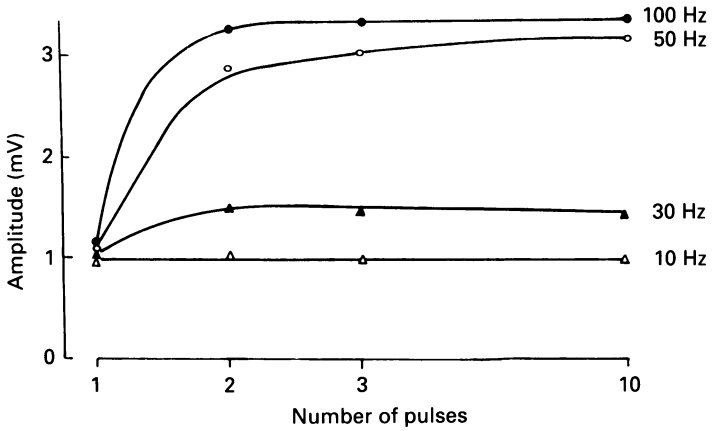

Fig. 3. Effects of pulse number and frequency on the amplitude of the non-quantal release. Mean values of three to five recordings are given. The S.E.M.s were similar to those in Fig. 2.

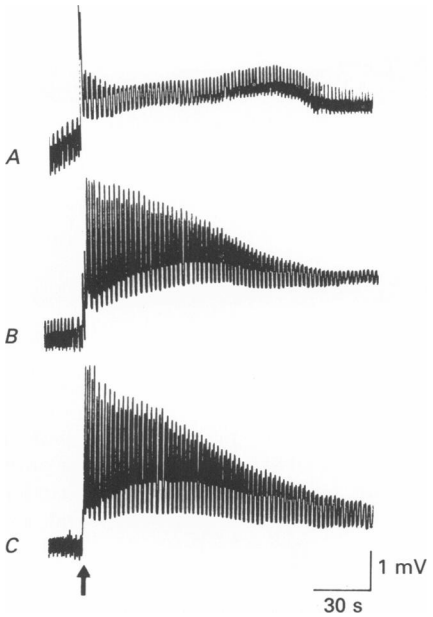

Fig. 4. Time courses of the non-quantal release evoked by nerve stimulation (arrow): 1 (*A*), 2 (*B*) or 10 (*C*) pulses at 100 Hz. The experiments were performed 10–60 min after wash-out of anti-ChE.

Effect of stimulation of the nerve on non-quantal release

In nerve–muscle preparations incubated for 2–5 h before addition of the anti-ChE, non-quantal release was not produced by the addition in about 70% of the muscles. Stimulation of these preparations, as well as of preparations where the non-quantal

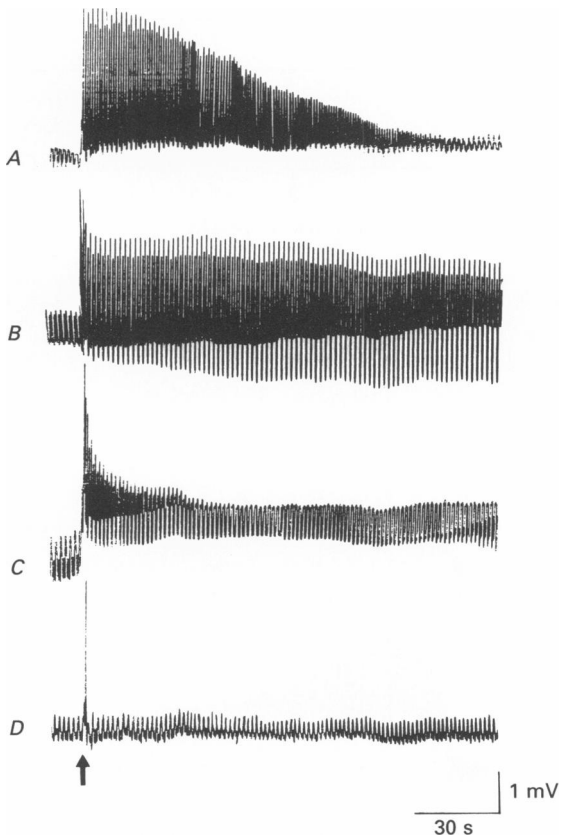

Fig. 5. Effects of several drugs on the non-quantal release evoked by nerve stimulation (arrow) with two pulses at 100 Hz. Control record (*A*) was obtained from the muscle 10 min after anti-ChE wash-out. Ouabain (10^{-4} M) (*B*), vesamicol (5×10^{-7} M) (*C*) and *d*-tubocurarine (10^{-4} M) (*D*) were applied 10 min before and were present during the measurements.

release had disappeared spontaneously during periods of 10–60 min after wash-out of the anti-ChE, produced a pronounced non-quantal release (Fig. 1*B*). The amplitude and the half-decay time of the stimulation-evoked non-quantal release was comparable with the first non-quantal release which occurred spontaneously.

The correlation between the amplitude of the non-quantal release after wash-out of the anti-ChE and the stimulation frequency was examined by changing the frequency (ten stimuli in all trials). The amplitude of the non-quantal release increased with the frequency of stimulation from 30 to 50 Hz, where it reached a maximum (Fig. 2). In other experiments, reduction of the number of stimuli was found to have little effect and two stimuli were still effective in evoking non-quantal

release. However, one stimulus was always less effective than two stimuli at a frequency of 50 or 100 Hz; at lower frequencies the effect of one stimulus was not much different from that of two or more stimuli (10 or 30 Hz; Figs 3 and 4).

Effects of drugs

Several drugs which are known to modify the quantal release of ACh have been tested for their effect on the amplitude and time course of the decline of the non-quantal release. The non-quantal release was initiated by stimulation of the nerve with two impulses at 100 Hz, 10–60 min after wash-out of the anti-ChE.

Ouabain is known to increase the frequency of spontaneous miniature endplate potentials and to enhance the non-quantal release measured intracellularly (Vyskočil & Illés, 1977, 1978; Vizi & Vyskočil, 1979). Ouabain (10^{-4} M) was without effect on the amplitude of the stimulus-evoked non-quantal release, but the half-time ($t_{\frac{1}{2}}$) of the decay of the response was increased about twofold (Table 1 and Fig. 5*B*).

Atropine, a specific muscarinic receptor blocker, at the concentration of 10^{-6} M decreased the amplitude and shortened $t_{\frac{1}{2}}$. Another muscarinolytic drug pirenzepine (10^{-7} and 10^{-6} M) reduced the amplitude and shortened $t_{\frac{1}{2}}$ similarly. The inhibitory effects of atropine (10^{-6} M) and pirenzepine (10^{-7} M) on the amplitude of non-quantal release persisted in the presence of ouabain but these drugs did not affect the prolongation of non-quantal release by ouabain (Table 1). The specific muscarinic agonist oxotremorine (10^{-8} M) enhanced the amplitude by 70% without any effect on $t_{\frac{1}{2}}$. Vesamicol, an inhibitor of the vesicular ACh transport system, at a concentration of 5×10^{-7} M, which has been already reported to inhibit non-quantal release determined intracellularly (Edwards *et al.* 1985; Vyskočil, 1985), decreased the maximum amplitude and $t_{\frac{1}{2}}$ by about half (Fig. 5*C* and Table 1). Vesamicol had no effect on the $t_{\frac{1}{2}}$ of the prolonged non-quantal release in the presence of ouabain (Table 1).

In the presence of *d*-tubocurarine (10^{-4} M) nerve stimulation did not depolarize the endplate zone; in fact, a small hyperpolarization developed which persisted up to the end of the experiment (Fig. 5*D*). This indicates that the non-quantal release could not be measured when the postsynaptic ACh receptors were blocked (cf. Vyskočil & Illés, 1977).

DISCUSSION

The data reported here are consistent with the proposal that non-quantal release is due to the incorporation of the vesicular ACh transport system into the plasma membrane of the nerve terminal during the exocytotic release of vesicle contents produced by activity of the nerve (Edwards *et al.* 1985, 1988). The spontaneous appearance of non-quantal release coincided closely with the appearance of muscle twitching due to the bursts of action potentials observed within seconds after addition of anti-ChE (Aizenman, Bierkamper & Stanley, 1986). Non-quantal release was absent in the presence of tetrodotoxin or a local anaesthetic which presumably blocked the generation of action potentials in the nerve.

There are other observations that also suggest a close correlation between quantal and non-quantal release. After denervation, the decrease of non-quantal release

coincides in time with the fall of spontaneous quantal release in the rat diaphragm (Zemková, Vyskočil & Edwards, 1987). Further, no changes in non-quantal release are seen when frog (Katz & Miledi, 1981) or mouse (Vyskočil *et al.* 1983) muscle nerves are stimulated tetanically in Ca^{2+}-free solution, i.e. under conditions when evoked quantal release is blocked (del Castillo & Katz, 1954).

In this respect it has to be stressed that the prolonged local endplate depolarization cannot be due to quanta of ACh released by nerve activity and persisting at the synaptic cleft for the following reasons. (a) Atropine (10^{-6} M) does not block spontaneous antidromic activity in the phrenic nerve in preparations treated with anticholinesterase (Aizenman *et al.* 1986), but it blocks local depolarization in our study; (b) prolonged endplate depolarization is completely inhibited by 3 mM-Mg^{2+} (Zemková & Vyskočil, 1989) while evoked quantal release is known to be inhibited at much higher concentrations of Mg^{2+} (5–10 mM, Jenkinson, 1957); (c) an increase in MEPP frequency does not appear after nerve stimulation with single pulses but only following 1 s tetanic stimulation (Zengel & Magleby, 1981). The frequency of MEPPs in anti-ChE-treated mouse diaphragm is maximally 7–10 Hz (mean 2·5 Hz, Zemková & Vyskočil, 1989) and their tails do not cause permanent depolarization of fibres.

In the present study, the amplitude of the non-quantal release elicited by nerve stimulation with two pulses was higher than with one pulse if the interval between the two pulses was no longer than 30 ms, i.e. if the nerve was stimulated with a frequency above 30 Hz. This indicates that the amplitude of the non-quantal release may be controlled by the number of carriers incorporated into the nerve terminal membrane. We found that non-quantal release was inhibited by muscarinolytic drugs atropine and pirenzepine and its amplitude was enhanced by the muscarinic agonist oxotremorine. That is, there may be a modulation by an interaction of the ACh accumulated within the subsynaptic space with the presynaptic nerve terminal receptors (Edwards *et al.* 1988). The results suggest that muscarinic receptors of the M_1 type, which are known as high-affinity pirenzepine receptors (Hammer, Berrie, Birdsall, Burgen & Hulme, 1980), may be involved in this modulation.

The non-quantal release decreased with time and disappeared at about 2–5 min after stimulation. It is unlikely that the disappearance was due to desensitization of presynaptic muscarinic receptors, since the rate of desensitization depends on agonist concentration and it would therefore be accelerated after ten stimuli as compared to two pulses.

A probable explanation for the decline of the non-quantal release would be the progressive reduction in the number of ACh carriers in the terminals by endocytosis. However, little is known about the time course of this process. Botulinum toxin, which binds to the terminal membrane, is taken up within about 20 min (Dolly, Black, Williams & Melling, 1984). Ouabain, which prolonged the duration of non-quantal release in our experiments, impairs endocytosis in the frog nerve terminal by overloading of the nerve terminal with vesicular membranes (Haimann, Torri-Tarelli, Fesce & Ceccarelli, 1985). The effect of ouabain on the time course of non-quantal release was not antagonized by vesamicol or muscarinic blockers though these drugs reduced the amplitude of non-quantal release in the presence of ouabain. We assume that the time course and the duration of non-quantal release were

determined by the rate of turnover of the vesicular ACh transport system in the nerve terminal and that the amplitude of non-quantal release was controlled by the activity of the ACh transport system which was blocked by vesamicol (Anderson, King & Parsons, 1983) and modulated by presynaptic muscarinic receptors.

The present results also address the problem of the source of the ACh for non-quantal release. It has been suggested that non-quantal release recruits from slowly (hours) accumulated ACh ('surplus') after anti-ChE treatment (Molenaar, Oen, Polak & van der Laaken, 1987). The rapid onset of non-quantal release during the first 60 s after anti-ChE application clearly demonstrates that this surplus ACh does not serve as a source for non-quantal release. The ACh required for non-quantal release is apparently available within seconds and its release might therefore take place under normal conditions when cholinesterase is active and when ACh carriers from vesicles are incorporated into nerve terminals during synaptic activation.

The present data indicate that non-quantal release is not due to a constant leakage of ACh from the nerve terminal. It is relatively small at rest when it is determined by the rate of spontaneous release of quanta but it may be extremely large immediately after nerve stimulation when hundreds of quanta are synchronously released.

It is well known that resting quantal release forms only a few per cent of the total release measured biochemically, the main release at rest being non-quantal (e.g. Vizi & Vyskočil, 1979). In the biochemical experiments (Doležal, Vyskočil & Tuček, 1983), neuromuscular preparations are incubated for at least 1 h in a small volume (about 1 ml). It is possible that under these conditions persisting nanomolar concentrations of ACh (Edwards *et al.* 1985) prevent the subside of non-quantal release and cause its permanent reappearance.

Under physiological conditions, as a somewhat long-lasting consequence of quantal release, the non-quantal release represents a memory trace of quantal release in the nerve terminal and might play a role in post-stimulation phenomena such as post-tetanic potentiation of transmitter release.

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