Physiol. Res. 52: 475-480, 2003

Long Release Latencies are Increased by Acetylcholine at Frog Endplate

D. SAMIGULLIN[1], E. A. BUKHARAEVA[1,2], E. NIKOLSKY[1,2]
S. ADÁMEK[3], F. VYSKOČIL[4,5]

[1]Institute of Biochemistry and Biophysics, Russian Academy of Sciences, Kazan, [2]State Medical University, Kazan, Russia, [3]Third Surgical Department, First Faculty of Medicine, Charles University, Prague, [4]Department of Animal Physiology and Developmental Biology, Faculty of Sciences, Charles University, Prague, and [5]Institute of Physiology, Academy of Sciences of the Czech Republic, Prague, Czech Republic

Received November 27, 2002
Accepted February 20, 2003

Summary

Uni-quantal endplate currents (EPCs) were recorded extracellularly at the frog neuromuscular synapse and their latency dispersions expressed as P_{90} were estimated in the presence of acetylcholine. Stimulation-evoked EPCs with long release latencies increased in number when acetylcholine was applied. P_{90}, which is designated as the interval between the minimal synaptic delay and the time at which 90 % of all measured uni-quantal EPCs had occurred, was significantly and reversibly increased by 66% from 0.51 ms to 0.85 ms in the presence of $5x10^{-4}$ M acetylcholine. This indicates that the evoked release pattern is less synchronous and the increased asynchrony leads to a substantial drop (by 28%) in the amplitude of reconstructed multi-quantal currents.

Key words

Quantal release • Synaptic latency • Acetylcholine • Model endplate current

Itroduction

According to the common model of synaptic transmission, the endplate currents are the result of a simultaneous secretion of many neurotransmitter quanta in response to a single nerve spike. Katz and Miledi (1965a,b) demonstrated a considerable variability in synaptic latencies for uni-quantal endplate postsynaptic currents (EPCs). Using mathematical modeling of uni-quantal EPC summation process, it has been suggested (Souček 1971, Giniatullin *et al.* 1995, Zefirov and Gafurov 1995) that the higher degree of synchrony can potentiate the synaptic transmission even when other parameters of postsynaptic responses are unchanged. Indeed, some compounds, noradrenaline in particular, can substantially shorten the release time of evoked quantal release when present in the muscle bath. This results in an increase of the amplitude of reconstructed multi-quantal EPCs up to 130 %. Thus, noradrenaline facilitates synaptic transmission by making the release of quanta more synchronous (Bukharaeva *et al.* 1999, 2000). On the contrary, it is known that another common neuro-transmitter acetylcholine (ACh) decreases the amplitude of evoked EPCs (Ciani and Edwards 1963) at the neuromuscular junction and this reduced amplitude can,

Fax +420 241 062 164
E-mail: physres@biomed.cas.cz http://www.biomed.cas.cz/physiolres

at least partly, be due to changes in the time pattern of quanta release.

In the present report we investigated the ACh effect on the time course of the quantal release and have demonstrated that ACh acts in a reverse manner than noradrenaline, i.e. it increases the dispersion of the synaptic latencies at frog neuromuscular junction. This markedly decreases the amplitude of reconstructed multi-quantal EPCs.

Methods

Animals and drug

Experiments were performed on isolated m. cutaneus pectoris neuromuscular preparations from the frog *Rana ridibunda* during winter period (January-February). Animals were anesthetized with ether before being stunned and pithed (Vyskočil *et al.* 2001). The muscles were pinned to the bottom of a 1.5 ml translucent chamber, superfused with the following low-Ca, high-Mg solution (mM): NaCl 113.0, KCl 2.5, $CaCl_2$ 0.4, $NaHCO_3$ 3.0, $MgCl_2$ 4.0; pH was adjusted to 7.3. The solution flowed through the muscle chamber at the rate of 3 ml/min. Monitoring of the bath solution throughout the experiment did not reveal any changes in pH after passing through the muscle chamber. Peltier semiconductor device controlled the temperature, the experiments being performed at 20.0±0.3 ^{o}C.

Acetylcholine chloride was from Sigma (St. Louis, MO, USA). The freshly dissolved drug was added to the superfusing solution. The measurements started 45 min after drug application. In most cases, the drug was washed out for another 60 min and EPCs were recorded again.

Electrophysiology

Suprathreshold stimuli of 0.1 ms duration were applied to the nerve at 2 s intervals through a pair of platinum electrodes located in a small adjacent moist chamber. This arrangement minimized the stimulus artifact. Extracellular nerve action currents and extracellular endplate currents were recorded using Ringer-filled pipettes with tip diameter of 2-3 μm and 1-3 MΩ resistance. Pipettes were positioned under visual control in the distal endplate region of a nerve terminal, 80-100 μm from the end of the myelinated segments of the axon, where quanta release are synchronous *per se* (Bukharaeva *et al*. 2002) and where – according to preliminary experiments – changes upon ACh application can be well demonstrated.

The recorded signals were filtered between 0.03 Hz to 10 kHz, digitized at 3-μs intervals by an analog-digital 9 bit-converter, fed into the computer and processed by our application program package.

By convention (Katz and Miledi 1965a,b, Barrett and Stevens 1972, Bukharaeva *et al.* 1999, 2001), the amplitudes of extracellular responses (transmembrane currents) are expressed in mV.

Uni-quantal endplate currents are required for correct latency measurements (Katz and Miledi 1965, Barrett and Stevens 1972, Bukharaeva *et al.* 2000). Experiments were therefore carried out in the presence of 0.4 mM Ca^{2+} and 4.0 mM Mg^{2+} (Bukharaeva *et al.* 2000). The quantal contents (m_o) of the low-quanta EPCs were determined from five or six stimulation periods (256 stimuli each) in the absence and in the presence of particular drugs. The number of failures in series of 250-400 uni-quantal responses were measured and m_o calculated as equal to $\ln N/n_o$, where N is the total number of stimuli and n_o is the number of failures (Del Castillo and Katz 1954, Martin 1955). Latency was measured as the time interval between the peak of the inward presynaptic Na^+ currents (NS in Fig. 1) and the time at which the rising phase of the quantal event reached 20 % of maximum. Because the amplitude of Na^+ current decreases along the nerve terminal (Mallart 1984, Shakiryanova *et al.* 1994), the distal recording site was selected so that the sodium peak was still clearly seen on averaged records. The limit of the latency measurement was 6 ms for experiments. Only data sets in which the nerve terminal spike changed by less than 5 % during the drug application and washout were analyzed further (Bukharaeva *et al.* 1999).

Statistical analyses of postsynaptic events were performed using Student's t-test for paired data, by multiple comparisons using the Windows Origin 6.1 program. Differences between two groups were considered statistically significant at the probability level P=0.05. The symbol n indicates the number of endplates measured in each group.

Cumulative curves were used to characterize quantitatively the changes in the time course of evoked secretion. They were constructed from latency histograms of the recorded uni-quantal EPCs (for details see Bukharaeva *et al.* 1999, 2002). The mean value of the shortest 5 % of latencies in each series was taken as the minimal synaptic delay. For comparison of dispersion histograms, mean minimal synaptic delay was subtracted before latency histograms were constructed. The interval between the minimal synaptic delay and the time at which

90 % of all measured uni-quantal EPCs had occurred was designated as P_{90}. The statistical significance of the difference between two cumulative curves was assessed by the Kolmogorov-Smirnov statistic, $p<0.05$ was taken as significant (Bronstein and Semendjaev, 1986; Van der Kloot, 1991, Bukharaeva *et al.* 1999, 2000, 2002).

To estimate the impact of transmitter release synchrony under the effect of ACh on multi-quantal EPC parameters, the comparison was carried out between the reconstructed EPCs in control and ACh-treated endplates. The EPCs were reconstructed using convolution method (Van der Kloot 1988) on the basis of experimentally obtained uni-quantal EPCs and their real latencies obtained in control and in the presence of ACh.

Results and Discussion

Quantal content and time course of evoked quantal secretion in control and in the presence of acetylcholine

In a low-Ca^{2+}, high-Mg^{2+} solution the mean quantal content (m_o) was 0.43±0.09 (n=12). The EPCs appeared after synaptic latencies which are dispersed during several milliseconds (Katz and Miledi 1965, Minenko and Magazanik 1986) as is documented by the native records in Figures 1A, 1B and 1C. The minimal synaptic latency was 0.32±0.02 ms (n=12) as reported earlier (Bukharaeva *et al.* 2002, Samigullin *et al.* 2003). The synaptic latency distribution histograms (not shown) were asymmetrical due to a certain incidence of long synaptic delays. The latency dispersion expressed as P_{90}, corrected for mean minimal synaptic latency (which was subtracted from each record) (Fig. 1D), was 0.51±0.06 ms for all control data which had been pooled (n=12). ACh was added to the superfusing solution in the concentration of $5x10^{-4}$ M. This concentration was used on the basis of preliminary tests of concentrations ranging from $1x10^{-7}$ to $1x10^{-3}$ M as optimal, which is effective but does not yet desensitize the postsynaptic receptors below the recognition threshold during the EPC recording. High concentration ACh was also used because of the activity of synaptic cholinesterase, which rapidly hydrolyzes ACh, and may unpredictably change the actual concentration of ACh in the cleft.

After application of ACh, the mean quantal content decreased to 0.12±0.08 (n=12, $p<0.05$) and the dispersion pattern substantially changed, mainly due to the increasing number of latencies longer than 1 ms (Fig. 1B). The control value of corrected P_{90} was 0.51±0.06 ms and it was significantly increased (by Kolmogorov-Smirnov criteria) by 66 % to 0.85±0.12 ms (mean ± S.E.M., n=6, $P<0.05$) in the presence of $5x10^{-4}$ M ACh. (Fig. 1D). After washing out the drug for 60 min, P_{90} decreased to 0.65±0.09 ms (n=4, $P<0.05$) which was not significantly different from the control. We have preliminary evidence (data not given) that the presynaptic action of ACh is mediated by both principal classes of cholinergic receptors. Nicotinic type regulates apparently the release dispersion as well as the number of quanta release (quantal content m_o). Muscarinic receptors can regulate m_o but not the release dispersion (cf. Švandová *et al.* 2001).

Postsynaptic effects

It is known that ACh in the concentration of $5x10^{-4}$ M decreases the uni-quantal postsynaptic currents to approximately 50% (from 0.28±0.01 mV in control to 0.15±0.01 mV after ACh, n=12, $p<0.05$), due to the combined effects on the postsynaptic receptors and resting potential of the postsynaptic membrane (Nikolsky *et al.* 1991, see the drop of EPC amplitudes in native recordings in Fig. 1B). The rise time of uni-quantal EPCs was not changed significantly (0.17±0.09 ms in the control and 0.16±0.06 ms after Ach, n=12, $p>0.05$). The time of decay of the uni-quantal EPCs was decreased from 1.51±0.31 ms to 1.03±0.12 ms (n=12, $p<0.05$). These postsynaptic effects are the result of an equilibrium between the depolarization of resting membrane potential by about 21 % (approximately from 70 mV to 55 mV due to receptor activation) and the desensitization which represents an inactive receptor state developing during long-lasting agonist application (Magleby and Pallota 1981).

One possible explanation of the higher dispersion was based on the assumption that small-quantum size EPCs might be released with shorter latencies than high-quantum size EPCs (Van der Kloot 1991). After ACh, when all EPCs would become much smaller due a lower postsynaptic sensitivity, some small-quantum size EPCs could not be recorded and high-quantum EPCs with longer latencies would be preponderant. This possibility was excluded by measuring the size of short-latency EPCs released with the delays in the interval between 0.32 ms (minimal synaptic latency) and 0.5 ms and long-latency EPCs (released after more than 1.0 ms from minimal synaptic latency), The amplitude in both groups was found to be almost identical, 0.28±0.01 mV and 0.27±0.02 mV before ACh and 0.15±0.01 mV and 0.14±0.02 mV 45 min after ACh respectively (150 EPCs were recorded in three synapses, 50 in each group).

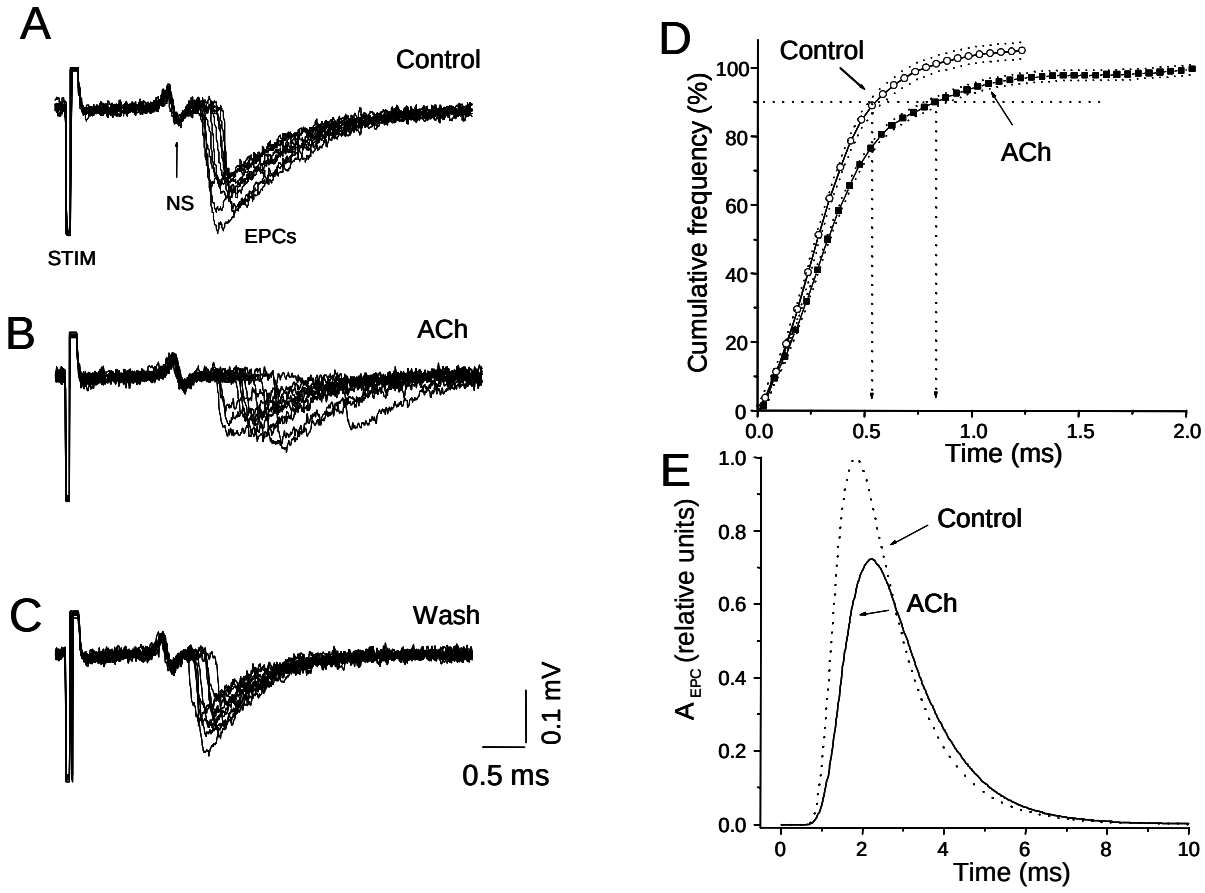

Fig. 1. *Effect of acetylcholine (ACh) on the latencies of quantal releases and on the amplitude of reconstructed multi-quantal EPC. A, B and C – 11 superimposed extracellular records taken from one endplate before (Control), after ACh application and after washout of drug (Wash). STIM. – stimulation artifacts, NS – extracellularly recorded presynaptic nerve spikes. The arrow indicates the Na^+ inward part of the presynaptic spike from which the latency of each EPC (downward deflections) was measured. D - the cumulative plots of latencies (abscissa, in ms) expressed as cumulative frequency (in % of total number from seven endplates, ordinate) are shown before (Control) and after ACh application. In D, the dotted lines parallel to the curves are confidence limits at P = 0.05. According to the Kolmogorov-Smirnov criteria, the difference between the curves is - significant if the confidence limits of the two curves do not overlap. The vertical dotted lines indicate the times when 90 % of the quanta have been released (P_{90} parameter). Note a significant increase of the release time after ACh. E – the effect of $5x10^{-4}$ M acetylcholine (ACh) on summed multi-quantal reconstructed endplate currents (EPCs). Summations of 30 real uni-quantal EPCs from single endplate was made and each latency was normalized according to the downward deflection of the peak of the inward presynaptic Na^+ currents before (dotted line) and during application of ACh (solid line). Amplitudes of the uni-quantal EPCs were normalized to exclude the contribution of postsynaptic decrease of EPCs in the presence of ACh. Ordinate - relative units, abscissa – time in ms.*

Modeling multi-quantal EPCs

Present data showed that ACh application desynchronizes the release of the neurotransmitter since this drug makes the release more dispersed in time. To quantify the impact of desynchronization on the EPC peak amplitude, multi-quantal EPCs were constructed by summation of the uni-quantal EPCs (their amplitudes were normalized to exclude the impact of a postsynaptic reduction of the uni-quantal signals) using real values of the synaptic delay of currents before and after ACh

(Fig. 1E). The amplitudes of the multi-quantal EPCs constructed from uni-quantal ones were smaller than those of the controls by as much as 28 % in the presence of desynchronizing ACh. Their rise time increased from 0.42 ms to 0.55 ms and decay times increased from 1.15 ms to 1.28 ms.

From the theoretical point of view, the present data concerning the substantial effects of ACh on transmitter quanta secretion synchrony and resulting multi-quantal EPC parameters suggest that more caution should be exhibited when calculating the quantal content of evoked synaptic currents by a direct method (i.e. dividing mean EPC by mean miniature EPCs, Del Castillo and Katz 1954, Martin 1955) because drugs that can affect secretion synchrony might make summation of uni-quantal responses non-linear (and thus wrongly interpreted) even when the subsynaptic membrane is voltage-clamped.

From the physiological point of view, increased asynchrony by acetylcholine released either quantally or non-quantally (Katz and Miledi 1977, Vyskočil and Illes 1977, Vyskočil *et al.* 1983, Mukhtarov *et al.* 1999, Vyskočil 2003) would have a significant physiological impact on cholinergic synapse because – as demonstrated here – it could induce a substantial drop in the amplitude of multi-quantal currents and thus hinder the synaptic transmission. Alternatively, this negative feedback might correct the abnormal increase of the quantal release when synapse is poisoned e.g. by potassium channel inhibitors such as aminopyridines or tetraethylammonium (Thesleff 1980).

Acknowledgements

We thank Dr. Pavel Hník for reading the manuscript and valuable suggestions. Supported by AV025011922, GAČR 305 1333, 202 1213, MŠMT 113100003 and RFBR 02-04-48901.

References

BARRETT EE, STEVENS CF: The kinetics of transmitter release at the frog neuromuscular junction. *J Physiol Lond* **227:** 691-708, 1972.

BRONSTEIN IN, SEMENDJAEV KA: *Handbook of Mathematics*. Publishing House „Science“, Moscow, 1986.

BUKHARAEVA E, KIM K, MORAVEC J, NIKOLSKY E, VYSKOČIL F: Noradrenaline synchronizes evoked quantal release at frog neuromuscular junctions. *J Physiol Lond* **517:** 879-888, 1999.

BUKHARAEVA E, SAMIGULLIN D, NIKOLSKY E, VYSKOČIL F: Cyclic AMP synchronizes evoked quantal release at frog neuromuscular junctions. *Physiol Res* **49:** 475-479, 2000.

BUKCHARAEVA, EA, SAMIGULLIN D, NIKOLSKY EE, VYSKOČIL F: Different regulation of quanta release by protein kinase A in proximal and distal parts of the frog muscle endplate. *Physiol Res* **50:** P5, 2001.

BUKHARAEVA E, SAMIGULLIN D, NIKOLSKY E, VYSKOČIL F: Protein kinase A cascade regulates quantal release dispersion at frog muscle endplate. *J Physiol Lond* **538:** 837-848, 2002.

CIANI S, EDWARDS C: The effect of acetylcholine on neuromuscular transmission in the frog. *J Pharmacol Exp Ther* **142**: 21-23, 1963.

GINIATULLIN R, KHEEROUG L, VYSKOČIL F: Modelling endplate current: dependence on quantum secretion probability and postsynaptic miniature current parameters. *Eur Biophys J* **23**: 443-446, 1995.

DEL CASTILLO J, KATZ B: Statistical factors involved in neuromuscular facilitation and depression. *J Physiol Lond* **124:** 574-585, 1954.

KATZ B, MILEDI R: The measurement of synaptic delay, and the time course of acetylcholine release at the neuromuscular junction. *Proc R Soc B Biol Sci* **161**: 483-495, 1965a.

KATZ B, MILEDI R: The effect of temperature on the synaptic delay at the neuromuscular junction. *J Physiol Lond* **181**:656-670, 1965b.

KATZ B, MILEDI R: Transmitter leakage from motor nerve endings. *Proc R Soc B Biol Sci* **196**: 59-72, 1977.

MAGAZANIK LG, MINENKO ML: Polymodality of the distribution of synaptic delays in the neuromuscular junctions of the frog /in Russian). *Neirofiziologiia* **18:** 748-756, 1986.

MAGLEBY K, PALLOTTA B: A study of desensitization of acetylcholine receptors using nerve-released transmitter in the frog. *J Physiol Lond* **316**: 225-250, 1981.

MARTIN AR:. A further study of the statistical composition of the endplate potential. *J Physiol Lond* **130:** 114-122, 1955.

MALLART A: Presynaptic currents in frog motor endings. *Pflügers Arch* **400**: 8-20, 1984.

MUKHTAROV MR, VYSKOČIL F, URAZAEV AK, NIKOLSKY EE: Non-quantal acetylcholine release is increased after nitric oxide synthase inhibition. *Physiol Res* **48:** 315-317, 1999.

NIKOLSKY E, BUKHARAEVA E, STRUNSKI E, VYSKOČIL F: Depression of miniature end-plate potential frequency by acetylcholine and its analogues in frog. *Br J Pharmacol* **104:** 1024-1032, 1991.

SAMIGULLIN D, BUKHARAEVA E, NIKOLSKY E, VYSKOČIL F: Temperature effect on proximal to distal gradient of quantal release of acetylcholine at frog endplate. *Neurochem Res* **28:** 507-514, 2003.

SHAKIRYANOVA D, ZEFIROV A, NIKOLSKY E, VYSKOČIL F: The effect of acetylcholine and related drugs on currents at the frog motor nerve terminal. *Eur J Pharmacol* **263**:107-114, 1994.

SOUČEK B: Influence of latency fluctuations and the quantal process of transmitter release on the end-plate potential´s amplitude distribution. *Biophys J* **11**: 127-139, 1971.

ŠVANDOVÁ I, VYSKOČIL F, UJEC E: NO-synthase inhibition partially mimics postdenervation changes in rat skeletal muscles. *Physiol Res* **50:** 29, 2001.

THESLEFF S. Aminopyridines and synaptic transmission. *Neuroscience* **5**: 1413-1419, 1980.

VAN DER KLOOT W: Estimating the timing of quantal releases during end-plate currents at the frog neuromuscular junction. *J Physiol Lond* **402**: 595-603, 1988.

VAN DER KLOOT W: The regulation of quantal size. *Prog Neurobiol* **36:** 93-130, 1991.

VYSKOČIL F: Early postdenervation depolarization is controlled by acetylcholine and glutamate *via* nitric oxide regulation of chloride transporter. *Neurochem Res* **28**: 575–585, 2003.

VYSKOČIL F, ILLES P: Non-quantal release of transmitter at mouse neuromuscular junction and its dependence on the activity of Na^+-K^+ ATPase. *Pflügers Arch* **370:** 295-297, 1977.

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

VYSKOČIL F, GINIATULLIN RA, TALANTOVA M: Desensitization of acetylcholine nicotinic receptors in frogs Rana ridibunda and Rana temporaria. *Physiol Res* **50:** P33, 2001.

ZEFIROV AL, GAFUROV O SH: Analysis of pre- and postsynaptic factors of asynchronity of transmitter release and amplitude-temporal parameters of postsynaptic response in neuromuscular junction. (in Russian) *Neirofiziologiia* **27**: 163-170, 1995.

Reprint requests

Prof. MUDr. F. Vyskočil, DrSc, Institute of Physiology, Academy of Sciences of the Czech Republic, Vídeňská 1083, 142 20 Prague 4, Czech Republic, E-mail: vyskocil@biomed.cas.cz