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RAPID COMMUNICATION

Cyclic AMP Synchronizes Evoked Quantal Release at Frog Neuromuscular Junctions

E. BUKCHARAEVA[1,2], D. SAMIGULLIN[2], E.E. NIKOLSKY[1,2], F. VYSKOČIL[3]

[1]Kazan Medical University, Kazan, Russia, [2]Kazan Institute of Biochemistry and Biophysics, Russian Academy of Sciences, [3]Institute of Physiology, Academy of Sciences of the Czech Republic, and Department of Animal Physiology and Developmental Biology, Charles University, Prague, Czech Republic

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Summary

At frog neuromuscular junction, noradrenaline (NA) shortens the release period for evoked quantal release acting on a β1 receptor. To test the hypothesis that this action of NA is mediated by cAMP, we measured the latencies of focally recorded uni-quantal endplate currents (EPCs) after application of dibutyryl-cAMP (db-cAMP) and adenylyl cyclase activator, forskolin. The interval between the time when responses with minimal delay appeared and the point at which 90 % of all latencies had occurred (P_{90} parameter) was shortened in the presence of both $1x10^{-6}$ mol/l db-cAMP and $1x10^{-6}$ mol/l forskolin by about 30 %. The cAMP-induced shortening is equal to that found after application of NA and effects of both drugs are not additive.

Key words

Acetylcholine • Frog muscle • cAMP • Quantal release

Introduction

The intensity and time course of acetylcholine (ACh) release can modulate both presynaptic and postsynaptic parts of signal transmission from nerve to muscle (e.g. Bukcharaeva *et al.* 1999a, Giniatullin *et al.* 1999, Mukhtarov *et al.* 1999, Nikolsky *et al.* 1999, Urazaev *et al.* 1997, 1998). In this respect, we studied recently the effect of noradrenaline (NA) on ACh release with the aim to explain the neuromuscular facilitation by some catecholamines observed many years ago (Orbeli 1923). We found that the interval between the time when responses with minimal delay appeared and the point at which 90 % of all latencies had occurred was shortened in the presence of $1x10^{-5}$ mol/l NA by about 35 %. This suggests that NA facilitates synaptic transmission by making the release of quanta more nearly synchronous. Inhibitor and agonist experiments showed that NA acts on β_1 receptor and computer modeling demonstrated that better synchronization of release significantly increased

Fax+4202 24920590
E-mail: physres@biomed.cas.cz
http://www.lf2.cuni.cz/physiolres/physiolres.htm

the size of reconstructed multi-quantal EPCs. (Bukharaeva *et al.* 1998, 1999b).

In the present report we measured the latencies of focally recorded uni-quantal endplate currents (EPCs) after application of permeable analogue of cyclic AMP, dibutyryl-cAMP (db-cAMP) and adenylyl cyclase activator, forskolin to demonstrate that this action of NA on β1 receptors is mediated by cAMP (Sutherland and Robinson 1966, Greengard and Kebabian 1974, Dryden *et al.* 1988).

Material and Methods

Experiments were performed on isolated m. cutaneus pectoris neuromuscular preparations from the frog *Rana ridibunda* during winter period (October-March). Animals were anesthetized with ether before being stunned and pithed. The isolated preparations were pinned on the bottom of a 3.5 ml translucent chamber with several compartments, superfused with the following solution (mmol/l): NaCl 113.0, KCl 2.5, $CaCl_2$ 0.2, $NaHCO_3$ 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. Temperature was controlled by a Peltier semiconductor device. The experiments were carried out at 20.0±0.3 °C.

The drugs, noradrenaline, db-cAMP and forskolin (all from Sigma, St. Louis, MO, USA) were added to the solution and measurements were usually started 20-40 min after drug application. In most cases, drugs were washed out for another 60 min and EPCs were recorded again. Suprathreshold stimuli of 0.1 ms duration were delivered to the nerve at 2 s intervals *via* platinum electrodes. Nerve action potentials and extracellular endplate currents were recorded using focal extracellular pipettes with tip diameter of 2-3 μm and 1-3 MΩ resistance, filled with 0.2 mol/l Ca^{2+} Ringer solution. Pipettes were positioned under visual control in the proximal endplate region of a large nerve terminal, 10-15 μm from the beginning of the myelination of the axon, where three-component nerve spikes, EPCs and miniature EPCs can be recorded (Mallart 1984, Shakiryanova *et al.* 1994). These signals were filtered between 0.03 Hz to 10 kHz and processed by computer. Amplitudes of extracellular responses are expressed in mV.

To estimate the time course of individual quantal releases from the dispersion of synaptic delay values, uni-quantal endplate currents were recorded in the Ringer solution with 0.2 mol/l Ca^{2+} and 4.0 mol/l Mg^{2+}. The number of failures in stimulation periods of 250-400 uni-quantal responses were measured and the quantal content (m_0) was calculated as equal to $ln\ N/n_o$, where N is total number of stimuli and n_o is number of failures (Martin 1955).

Latency was measured as the time interval between the peak of the inward presynaptic Na^+ currents and the time at which the rising phase of the quantal event reached 20 % of maximum. The mean value of the shortest 5 % of latencies in each series was taken as the minimum synaptic delay. Statistical analyses of pre- and postsynaptic events were performed using Student's t-test for paired data (Microcal Origin).

The quantitative characteristics of the change in the time course of evoked secretion produced by action of NA, db-cAMP and forskolin were obtained by cumulative curves. They were built from latency histograms of the uni-quantal EPCs. The interval between the minimum synaptic delay and the time at which 90 % of all measured uni-quantal EPCs had occurred was designated as P_{90} parameter. The statistical significance of the difference between two cumulative curves was assessed by the Kolmogorov-Smirnov statistics, $p<0.05$ was taken as significant (Bronstein and Semendjaev 1986, Van der Kloot 1991).

Results and Discussion

Superimposed nerve action potentials and EPCs in response to stimulation under control conditions at 20 °C are shown in Figures 1A and 1C. The EPCs appear after the synaptic delay or latency (Katz and Miledi 1965). NA and db-cAMP were then applied at a concentration of $1x10^{-5}$ mol/l and $1x10^{-6}$ mol/l. These concentration were selected from preliminary tests of concentrations ranging from $1x\ 10^{-6}$ to $2x10^{-4}$ mol/l.

Noradrenaline. In the low Ca^{2+}, high-Mg^{2+} solution mean quantal content (m_0) was 0.33±0.09 (n=9). After NA ($1x10^{-5}$ mol/l) application it was 0.39±0.08 (n=9, P>0.05). The mean frequency of miniature EPCs (mEPCs) in the presence NA (1.73±0.45 per sec, n=9, P>0.05) was very similar to that recorded in controls before the drug (1.4±0.50 per sec). NA did not change the presynaptic action potential, the EPC amplitude and minimal synaptic delay (Bukharaeva *et al.* 1999b). NA did, however, change the latency distribution. To quantify the effects of NA on the synaptic latency, normalized cumulative curves of synaptic delays (Fig. 1B) for uni-

quantal EPCs before and after the addition of NA were constructed. The control value of P_{90}, 2.16±0.23 ms, was significantly reduced by 35 % (by the Kolmogorov-Smirnov criteria) to 1.41±0.10 ms (n=9, P<0.05, Fig. 1B) in the presence of NA.

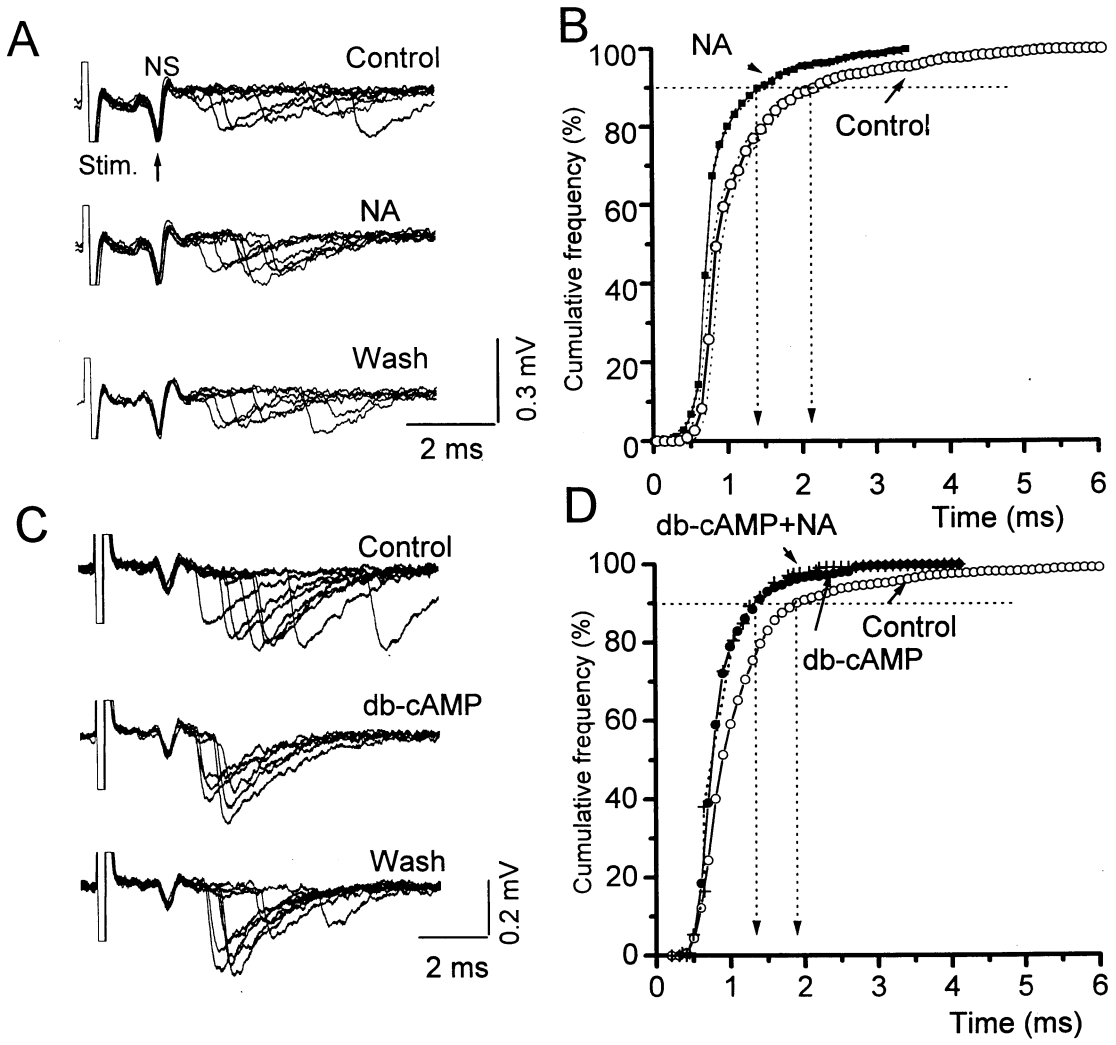

Fig. 1. *Effect of noradrenaline (NA) (A, B) and db-cAMP (C, D) on the latencies of quantal releases. In A and C, 6-7 superimposed extracellular records are shown which were taken from two endplates before (Control), after NA and db-cAMP application and after washout of drugs (Wash). Stim. – stimulation 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. In B and D, the cumulative plots of latencies (abscisae, in ms) expressed as cumulative frequency (in % of total number of EPCs recorded from seven endplates, ordinate) are shown before (Control), after noradrenaline (NA) and after db-cAMP application (for details see Bukharaeva et al. 1999b). In B, 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 statistically significant if the confidence limits of the 2 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 decrease of the release time after NA and db-cAMP, which is not cumulative (cAMP+NA, crosses, in D).*

db-cAMP. In similar way as NA, db-cAMP markedly changed the latency distribution (Fig. 1C,D): the number of EPCs with longer synaptic latencies was decreased, but the minimal synaptic delay was unchanged (0.46±0.02 ms in 9 control experiments versus 0.43±0.04 ms in the presence of db-cAMP, P>0.05). Normalized cumulative curves of synaptic delays (Fig. 1D) showed that the control value of P_{90}, 1.94±0.23 ms, was

significantly reduced to 1.38±0.10 ms (n=6, P<0.05, Fig. 1B) in the presence of db-cAMP after 60 min. The ratio between the P_{90} value in db-cAMP versus that in controls was 0.72; this drug thus shortened the early release phase by 28 %.

No other effects of the drug were found. Quantal content m_0 was not changed in the presence of db-cAMP, m_0 being 0.34±0.15 (n=5) before and 0.35±0.25 (n=5) after $1x10^{-5}$ db-cAMP application and 0.36±0.17 (n=5) after wash out of db-cAMP for 90 min. The mean frequency mEPCs in the presence of $1x10^{-6}$ mol/l db-cAMP (0.83±0.45 per sec, n=9, P>0.05) was very similar to that recorded in controls before the drug (0.74±0.50 per sec). Similarly to NA, it did not change the amplitude and decay time of mEPCs, suggesting no effect on postsynaptic receptor sensitivity (data not given).

db-cAMP plus noradrenaline. When added after db-cAMP, NA did not further shorten the early release phase (Fig. 1D). The P_{90} was 1.38±0.10 ms in the presence of db-cAMP and 1.35±0.05 ms, (n=5, P>0.05) when NA was added. The absence of the effect can be expected if NA effect would be fully substituted by endogenous cAMP.

Forskolin. Adenylyl cyclase activator forskolin (Laurenza *et al.* 1987) was used in the concentration of $1x10^{-6}$ mol/l, which does not yet influence the miniature EPCs frequency and postsynaptic ACh sensitivity (Khirough *et al.* 1998). Time course of evoked quantal secretion in the presence of forskolin was almost equally changed as in the presence of db-cAMP. This is demonstrated by the reduction of P_{90} parameter from 1.85±0.12 ms in controls to 1.25±0.08 ms (n=6, P<0.05). The ratio between the P_{90} value in forskolin versus that in controls was 0.69 which means that forskolin shortened the early release phase by 32 %.

The results strongly suggest that NA facilitates synaptic transmission at frog neuromuscular junction by making the release of quanta more nearly synchronous through β1 receptors coupled with adenylyl cyclase and cAMP production. As phosphorylation target, N-type calcium channels involved in ACh release might be considered (Wessler *et al.* 1990a,b) or β_1 activation might serve as a substitute for the synchronizing role of external Ca^{2+} in Ca^{2+}-free medium. In fact, it has been suggested by Silinsky and Vogel (1986) that cAMP-dependent phosphorylation of Ca^{2+} storage sites could liberate Ca^{2+} into the nerve terminal cytoplasm. A better synchronization could then be expected. This mechanism should be apparently separated from the facilitatory effect of NA on ACh exocytosis measured as quantal number (Yawo 1996) and further analysis of phosphorylation-dephosphorylation cascades and targets is required. Physiologically, the synchronizing action of NA might potentiate neuromuscular transmission during transmitter exhaustion and at other extreme physiological states where the quantal content is reduced, such as survival in cold and hibernation, as well as during motor nerve regeneration.

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References

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

BUKCHARAEVA EA, KIM KK, NIKOLSKY EE, VYSKOČIL F: Synchronization of secretion of the evoked quanta transmitter as a mechanism of sympathomimetic facilitating action. *Russian Physiol J* **84**: 1121-1131, 1998.

BUKCHARAEVA E, IPATOVA T, NIKOLSKY EE, ŘÍČNÝ J, VYSKOČIL F: Carbachol and α-bungarotoxin decrease spontaneous quantal secretion at the frog neuromuscular junction. *Physiol Res* **48**: S60, 1999a.

BUKCHARAEVA EA, KIM KK, NIKOLSKY EE, MORAVEC J, VYSKOČIL F: Noradrenaline synchronizes evoked quantal release at frog neuromuscular junctions. *J Physiol Lond* **517**: 879-888, 1999b.

DRYDEN W, SINGHY, GORDON T, LAZARENKO G: Pharmacological evaluation of cyclic AMP and transmitter release at mouse neuromuscular junction. *Can J Physiol Pharmacol* **66**: 207-212, 1988.

GINIATULLIN R, TALANTOVA M, KRŮŠEK J. SHARIFULLINA E, VYSKOČIL F: Desensitization-promoting action of verapamil on nicotinic receptors expressed in COS cells and on frog endplate. *Physiol Res* **48**: S47, 1999.

GREENGARD P, KEBABIAN J: Role of cyclic AMP in synaptic transmission in the mammalian peripheral nervous system. *Fed Proc* **33**: 1059-1067, 1974.

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

KHIROUGH L, SOKOLOVA E, GINIATULLIN R, AFZALOV R, NISTRI A: Recovery from desensitization of neuronal nicotinic acetylcholine receptors of rat chromaffin cells is modulated by intracellular calcium through distinct second messengers. *J Neurosci* **18**: 2458-2466, 1998.

LAURENZA A, KHADELWAL Y, DE-SOUZA N, RUPP R, METZGER H, SEAMON K: Stimulation of adenylate cyclase by water-soluble analogues of forskolin. *Mol Pharmacol* **32**: 133-139, 1987.

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

MARTIN AR: A further study of the statistical composition of the endplate potential. *J Physiol Lond* **130:** 114-12, 1955.

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

NIKOLSKY EE, MUKHTAROV MR, URAZAEV AKh, VYSKOČIL F: Nitric oxide modulates the non-quantal acetylcholine release at the rat neuromuscular junction. *Physiol Res* **48**: S101, 1999.

ORBELI LA: Die sympatetische Innervation der Skelettmuskeln. *Bull Instit Sci Lesheften* **6:** 194-197,1923.

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

SILINSKY SM, VOGEL SM: The effects of an adenylate cyclase inhibitor on the electrophysiological correlates of neuromuscular transmission in the frog. *Br J Pharmacol* **88**: 799-805, 1986.

SUTHERLAND E, ROBINSON G: The role of cyclic 3', 5'-AMP in response to catecholamines and other hormones. *Pharmacol Rev* **18**: 145-188, 1966.

URAZAEV AKh, NAUMENKO NV, POLETAYEV GI, NIKOLSKY EE, VYSKOČIL F: Acetylcholine and carbachol prevent muscle depolarization in denervated rat diaphragm. *Neuroreport* **8**: 403-406, 1997.

URAZAEV AKh, NAUMENKO NV, NIKOLSKY EE, VYSKOČIL F: The effect of imidazole-containing compounds on the postdenervation decrease of membrane potential in the rat diaphragm. *Physiol Res* **47**: 291-295, 1998.

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

WESSLER I., DOOLEY DJ, OSSWALD H., SCHLEMMER F: Differential blockade by nifedipine and omega-conotoxin GVIA of alpha$_1$- and beta$_1$-adrenoceptor-controlled calcium channels on motor nerve terminals of the rat. *Neurosci Lett* **108**:173-178, 1990a.

WESSLER I, HOLZER G, KANSTLER A: Stimulation of β_1 adrenoreceptors enhances electrically evoked [H^3] acetylcholine release from rat phrenic nerve. *Clin Exp Pharmacol Physiol* **17:** 23-32, 1990b.

YAWO H: Noradrenaline modulates transmitter release by enhancing the Ca^{2+} sensitivity of exocytosis in the chick ciliary presynaptic terminal. *J Physiol Lond* **493:** 385-391, 1996.

Reprint requests

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