Role of Na^+/K^+ ATPase in Presynaptic Afteraction of Exogenous Acetylcholine in Rat Diaphragm

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A long-term increase in the amplitude and quantum composition of potentials from the end-plate was observed in isolated rat diaphragm with inhibited acetylcholinesterase during washing from acetylcholine. The amplitude and temporal parameters of the miniature potentials did not change, while their frequency decreased in the presence of acetylcholine and continued to fall during 1-2 h of washing. The addition of ouabain restored the frequency of these potentials; in the presence of ouabain acetylcholine had no effect on this parameter. Hyperpolarization of the extrasynaptic area of muscle fibers (2-3 mV) caused by acetylcholine is preserved during washing. Hyperpolarization was also observed against the background of tolbutamide but not in the presence of ouabain and heparin.

Key Words: *acetylcholine; neuromuscular transmission; ion pump; presynaptic release of neurotransmitter*

Acetylcholine (ACh) acts not only as a transmitter substance in cholinergic synapses but also influences various cell systems [1] and is involved in neuromuscular transmission [5,10,13,14]. Previously, we showed that tetanization of motor nerve and short-term addition of exogenous ACh result in a long-term restoration of contraction force of isolated rat diaphragm with inhibited acetylcholinesterase (fatigue caused by sporadic repeated stimulation [3,4]). It was suggested that such an increase in the muscle working capacity has a presynaptic nature, and Na^+/K^+ ATPase is a possible target of ACh in this effect [2]. There is controversy over the effects of ACh and cholinomimetics on the release of neurotransmitters from nerve endings in mammals [6,10,12-14], which hampers their extrapolation under our experimental conditions. This study is an attempt to check up the hypothesis that "afteraction" of ACh is of presynaptic nature and that Na^+/K^+ pump is involved in this effect.

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MATERIALS AND METHODS

Albino rats were used. Experiments were performed on isolated phrenicodiaphragmal preparations in bathing solution containing (in mM): NaCl 137, KCl 5, $CaCl_2$ 2, $MgCl_2$ 2, $NaHCO_3$ 24, NaH_2PO_4 — 1, and glucose 11. The solution was bubbled with carbogen (95% O_2 and 5% CO_2) at 28°C (pH 7.4-7.6). End-plate potentials (EPP) and spontaneous miniature EPP (MEPP) induced by nerve stimulation were recorded extracellularly using glass microelectrodes (tip diameter 2-4 μ) filled with normal saline. Membrane potentials of resting muscle fibers were recorded intracellularly with standard glass microelectrodes. For muscle immobilization upon EPP measurements, the magnesium concentration was raised to 12-15 mM. The nerve was stimulated via the electrode with rectangular 0.1-msec pulses of 3-5 threshold amplitude. The responses were digitized at a 40-50 μsec intervals and analyzed in a DVK-2 computer; 100 MEPP or 50 EPP were averaged. Acetylcholine (0.1 μM) was added for 15 min in the bathing solution or applied locally via a micropipette

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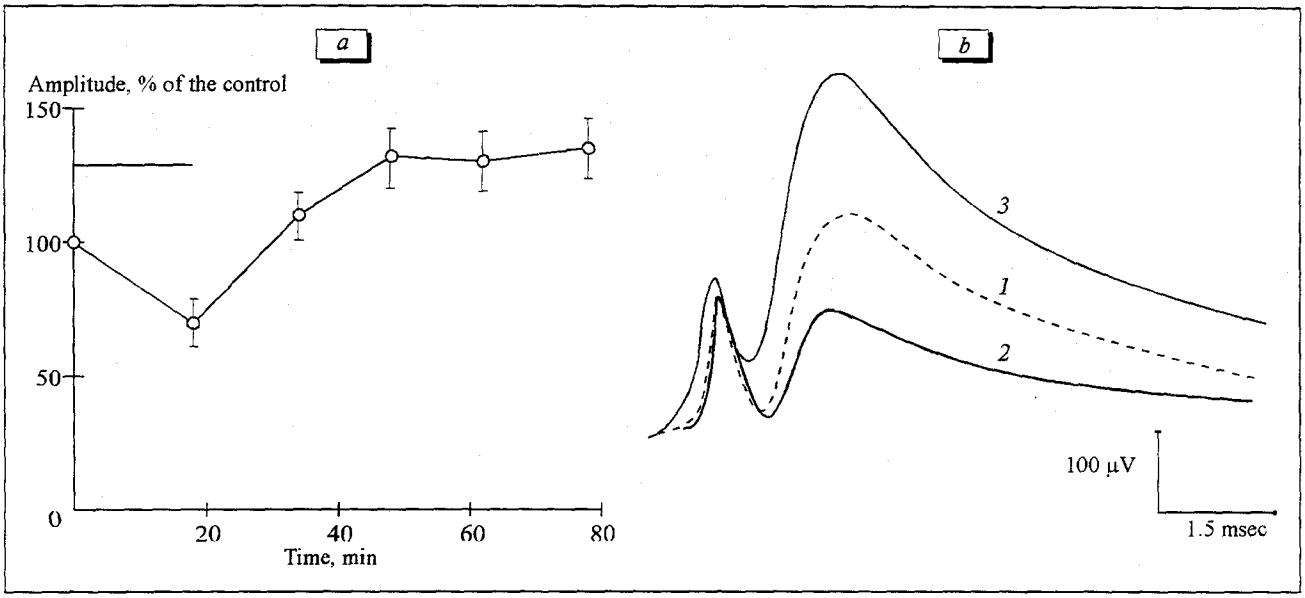

Fig. 1. Time course of end-plate potentials (EPP) in the presence of acetylcholine (ACh) and during its washing against the background of continuous stimulation at a frequency 1 pulse/sec (*a*); *b*) responses of end-plate and EPP in the control (*1*), 15 min after the addition of ACh (*2*), and after 60 min of washing (*3*). Here and in Figs. 2 and 3: horizontal lines indicate the presence of ACh in bathing solution.

(tip diameter 10-20 μ) during the same period. The phosphoorganic acetylcholinesterase inhibitor armine (0.4 μM) was added to the bathing solution throughout the entire experimental period 1 h after the diaphragm incision. Measurements were started 1 h after the addition of armine. Quantum composition (QC) of EPP was calculated by the coefficient of variation of their amplitudes. The responses recorded before the addition of ACh served as the control. The significance of differences was evaluated by Student's *t* test. The mean values are given in the text and figures.

RESULTS

Continuous stimulation at a frequency of 1 pulse/sec induced no significant changes in EPP. Acetylcholine added on the 30th-60th min of stimulation lowered EPP amplitudes after 15 min from 363±65 to 264± 60 μV (n=7). In all experiments, EPP amplitude increased after removal of ACh from bathing solution, reaching a plateau at a level of 478±89 μV (130.4±7.7% of the control, p<0.01) by the 30th min (Fig. 1). In some experiments, action potentials of muscle fibers were detected after 1 h of washing. MEPP were recorded before stimulation and immediately after its termination. The amplitude, duration of ascending phase, and the half-decline time of MEPP were, respectively, 143±41 μV, 0.299± 0.032 msec, and 1.04±0.11 msec prior to stimulation, and 137±18 μV, 0.303±0.033 msec, and 1.09±0.13 msec after stimulation, i.e., practically did not change. Calculations have confirmed that the EPP amplitude rise resulted from a QC increase: to 138% on the 30th min of washing and to 154% on the 60th min compared with the control.

The parameters (except frequency) of MEPP did not change significantly in the presence of ACh and during washing (Fig. 2), i.e., there were no substantial changes in the sensitivity of postsynaptic membrane.

The frequency of MEPP decreased to 63.3±9.2% of the initial value (1.4±0.2 msec, n=18) 15 min after the addition of ACh. The decrease was observed after removal of ACh from the bathing solution; and 1 h after washing the MEPP frequency was 12.3± 5.3% of the control (Fig. 3). The initial frequency

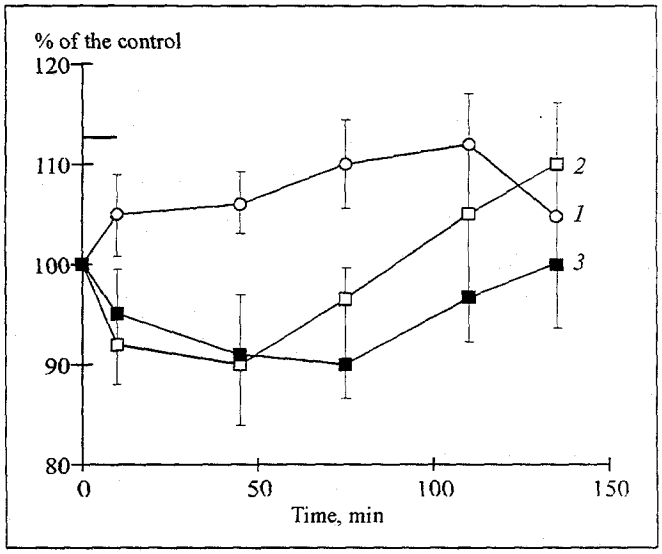

Fig. 2. Miniature end-plate potentials under the action of acetylcholine and during its washing. *1*) duration of ascending phase; *2*) half-decline time; *3*) amplitude.

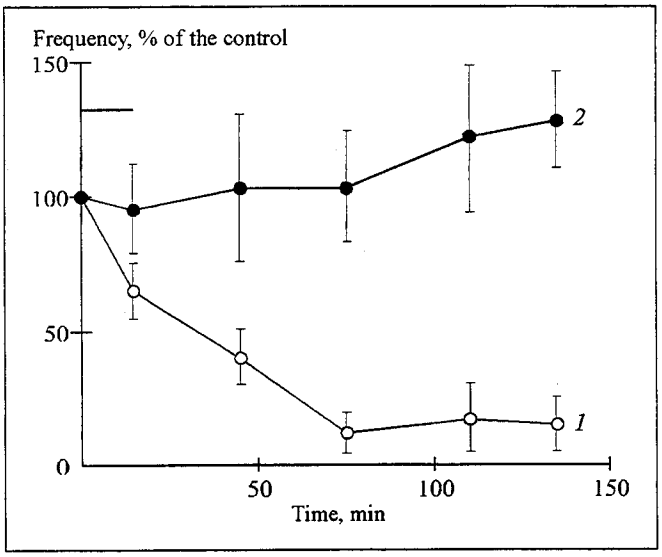

Fig. 3. Changes in the frequency of miniature end-plate potentials in the presence of ACh and during its washing in the absence (*1*) and presence (*2*) of ouabain.

of MEPP was restored 30 min after the addition of ouabain (10 μM) to the bathing solution. When ouabain was added together with armine, the MEPP frequency did not change significantly in the presence of ACh or after its removal (Fig. 3).

Resting potentials of muscle fibers were employed as an indirect indicator of the ion pump activity. Hyperpolarization in the extrasynaptic area of muscle fibers (2.0±0.6 mV, $p<0.01$) was observed 15 min after the addition of ACh. After removal of ACh, this hyperpolarization gradually increased, being 3.2±0.6 mV after 2 h of washing; resting potentials were recorded in 149-153 fibers in 7 muscles. The time course of this hyperpolarization coincided with that of MEPP frequency. Hyperpolarization was not observed in the presence of ouabain (20-50 μM). A similar hyperpolarization with a similar time course was observed in the presence of 100 μM tolbutamide, a cAMP-dependent protein kinase A blocker, but not in the presence of 1 μM heparin that blocks inositol tris-phosphate receptors and induces calcium mobilization from the intracellular stores.

The results of our experiments with ouabain and the finding that low concentrations of ACh activate Na^+/K^+-ATPase [7,8] suggest that ACh causes a long-term increase in the activity of this enzyme. If so, a decrease in the MEPP frequency with a simultaneous increase in the QC of EPP are due to hyperpolarization of nerve terminals caused by activation of their electrogenic pump or reduction in the extracellular potassium concentration associated with activation of the Na^+/K^+ pump in muscle fibers. This is indirectly confirmed by prolonged "trace" hyperpolarization of muscle fibers (2-3 mV), which is potentially much greater in nerve endings with a high input resistance.

Presumably, Na^+/K^+ ATPase is the target of ACh in the phenomenon of spontaneous and induced release of neurotransmitter, judging from these results, our previous findings [2,4], and similarity between the catalytic subunit of Na^+/K^+ ATPase and N-cholinergic receptor [9]. It should be noted that in the presence of ACh the amplitude of EPP decreased (Fig. 1), which is consistent with the presynaptic action of ACh according to the negative feedback principle [10,14]. Similar to autoreceptors, Na^+/K^+ ATPase may serve as a binding site for ACh but with an opposite sign of presynaptic action. In this case the resultant effect will depend on specific conditions, which may account for the controversy over the effect of ACh-induced neurotransmitter release [6,10,12-14].

Thus, the increase in the working capacity of exhausted muscles [2,4] is probably based on a long-term rise of QC and EPP. Na^+/K^+ ATPase may serve as a target for ACh, while the long-term "afteraction" of ACh may be due to a prolonged increase in the enzyme activity with participation of intracellular messengers. Na^+/K^+ ATPase can be regulated via several pathways, for example, by protein kinase C [11]. The results of the present study suggest that the observed effects are not associated with cAMP-dependent protein kinase A, but are coupled to intracellular calcium and inositol tris-phosphate.

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