Effect of Exogenous Acetylcholine on Neuromuscular Transmission in the Stimulated Rat Diaphragm

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Nerve stimulation is performed in rat phrenicodiaphragmal preparations with armine-inhibited acetylcholinesterase. Acetylcholine (1×10^{-7} M) is added to the saline for 15 min, and as it is washed off (during 1-2 h), the amplitude of isometric contractions and of the total action potential increases in the continuously stimulated muscle. Contractions in response to direct muscle stimulation remain unchanged. The membrane resting potential of muscle fibers exposed to acetylcholine shifts by 2-3 mV toward hyperpolarization and remains at this level for 2 h after the removal of acetylcholine from the saline.

Key Words: *acetylcholine; neuromuscular transmission; membrane potential*

Numerous studies show that the functions of exogenous acetylcholine (AC) in the neuromuscular synapse are not confined to simple signal transmission from nerve ending to muscle fiber. The possible contribution of AC transmitters to synaptic plasticity and adaptive changes has been discussed [1]. Previous studies [3] have demonstrated that in the rat diaphragmal preparation with inhibited acetylcholinesterase (ACE) which is continuously stimulated with single pulses, nerve tetanization results in a marked augmentation of subsequent single muscle contractions. This has been attributed to the accumulation of endogenous AC in the muscle, since such an effect is abolished by cholinolytics and is reproduced after a short-term addition of exogenous AC to the saline. In the present study we further explored this phenomenon.

MATERIALS AND METHODS

The experiments were carried out on isolated neuromuscular preparations of the rat diaphragm exposed to 4×10^{-7} M armine, an organophosphate inhibitor of ACE. We used physiological saline of the following composition (mM): NaCl 137, KCl 5, $CaCl_2$ 2, $MgCl_2$ 2, $NaHCO_3$ 24, NaH_2PO_4 1, glucose 11 (pH 7.4-7.6; 28°C). The saline was continuously aerated with carbogen (95% O_2 and 5% CO_2). Continuous nerve stimulation was performed via the absorbing electrode with 0.1-msec pulses (3-5 thresholds) at a frequency of 1 pulse/sec (60 cpm). Stimulation was started 1 h after the addition of armine to the solution (i.e., after ACE had been completely suppressed). Exogenous AC (10^{-7}M) and its agonists were added to the solution at the 30th min of tetanization for 15 min, after which they were removed from the solution. Tubocurarine, scopolamine, tolbutamide, or heparin used in the experiments were added simultaneously with armine and remained in the solution throughout the experiment. Muscle contractions were recorded in the isometric mode on a mechanotron. The total action potential (AP) was recorded with silver wire electrodes applied on and beneath the muscle to its nerve-free portion. The same electrodes were used for direct stimulation of the muscle with single 10-msec supramaximal pulses (nerve stimulation being discontinued for a while).

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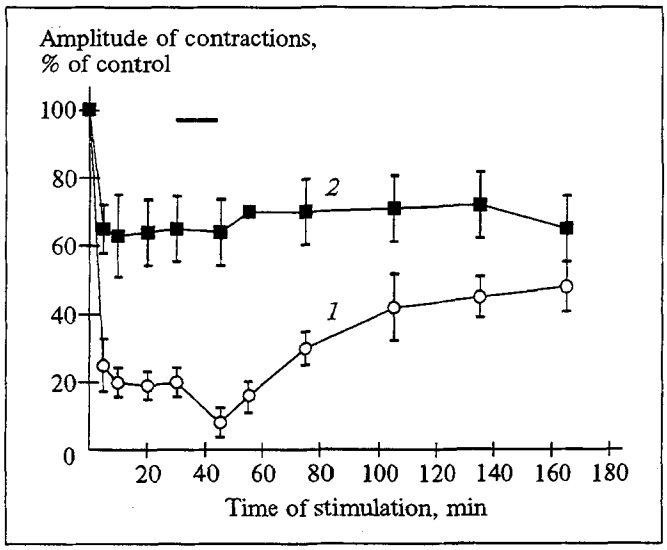

Fig. 1. Variation of amplitude of contractions in rat diaphragm for continuous nerve stimulation at 1 pulse/sec. Contractions in response to nerve stimulation (*1*) and to direct stimulation of muscle (*2*). Here and in Fig. 2: exposure to AC is marked by a horizontal line.

In a separate series of experiments the membrane resting potential (MRP) and AP during tetanization were recorded with glass microelectrodes in the nonsynaptic zone of the muscle fibers. When microelectrodes were implanted into the muscle fiber, nerve stimulation was discontinued. The parameters determined prior to tetanization were regarded as the control. The reliability of differences was assessed using Student's test; the results are presented as the sum of means and error of means.

RESULTS

The dynamics of muscle stimulation is shown in Fig. 1. At the 30th min of stimulation the amplitude of contractions in response to nerve tetanization was as low as 20.4±2.3% (n=13) of the control. Fifteen minutes after the addition of AC the amplitude of contractions dropped even lower (2-3-fold). As soon as the 10th-20th min after the removal of AC by washing of the muscle with the initial saline, the amplitude of contractions reverted to the level observed prior to the addition of AC, after which it gradually increased over 1-2 h (Figs. 1 and 2), attaining 49.3±6.8% of the control (p>0.99) after 2 h of washing. Such an increase was not observed in the presence of 1.5×10^{-8} M tubocurarine (n=5), a blocker of N-cholinoceptors, 10^{-7} M scopolamine (n=5), a blocker of M-cholinoceptors, as well as 10^{-4} M heparin (n=5), inhibiting calcium release from the intracellular depots (Fig. 2). Meanwhile, in the presence of 2×10^{-6}-2×10^{-4} M tolbutamide (n=5), an inhibitor of cAMP-dependent protein kinase A, an enhancement of contractions during AC washing off was clearly evident. The same effect was observed when 5×10^{-4} M dibutyryl cAMP (n=2) was added (without subsequent washing off) to the solution instead of AC.

During washing off, an augmentation of muscle contractions was also observed in the experiments (n=7), where 10^{-6}-10^{-5} M carbacholine, an AC analog, was used instead of AC, although in this case the enhancement of contraction was only half that after the addition of AC. However, when 10^{-6}-2×10^{-5} M oxotremorin, an agonist of M-cholinoceptors, was used (n=4), washing off did not result in the augmentation of contractions.

In the experiments with the use of AC changes of the amplitude of the total AP were similar to those of the amplitude of muscle contractions: during tetanization the amplitude decreased and during washing off it increased from 9.0±1.5 (at the 30th min of tetanization) to 19.0±1.5% of the control (after 2 h of AC washing off) (n=3).

As soon as the 5th min of tetanization the amplitude of contractions caused by direct stimulation of the muscle dropped 35% (n=7) and then remained unchanged for both the addition of AC and its removal from the saline (Fig. 1).

After the addition of AC to the saline the MRP reliably (p>0.99) dropped from -73.1±0.4 mV (number of fibers 158, number of muscles 6) at the 30th min of tetanization to -75.4±0.4 mV (number of fibers 147, number of muscles 6) at the 15th-20th min of exposure to AC. After the removal of AC from the solution a 2-3-mV hyperpolarization was preserved for 1.5 h: at the 60th min of washing off the MRP was -76.2±0.4 mV (number of fibers 159, number of muscles 6). The amplitude and duration of muscle fiber AP did not change reliably. In similar experiments without the addition of AC (5 muscles) we observed only a gradual depolarization of muscle fibers during tetanization.

The results obtained by recording the total and intracellular AP and by directly stimulating the muscle suggest that electromechanical transmission and the contractile apparatus do not contribute to the enhancement of contractions after the removal of AC. This effect may be attributed to the increased number of muscle fibers contracting in response to nerve stimulation. Evidently, during exposure to exogenous AC, some long-term processes involving intracellular transmitters are triggered, guaranteeing the continuation of synaptic transfer long after AC has been removed from the solution. The experiments with heparin, tolbuta-

mide, and dibutyryl cAMP suggest that such processes may be calcium-dependent, associated with the level of cAMP, but not mediated by cAMP-dependent proteinkinase A.

The mechanisms underlying the increased guarantee of synaptic transfer (pre- and/or postsynaptic) and target cells for AC in this mechanism are still unclear. AC is known to increase the spontaneous synthesis of ACE [1], but in our case this cannot result in the formation of free ACE in the synapse, due to the presence of armine in the solution. Presumably, the efficacy of synaptic transfer may be regulated via activation of the postsynaptic N-cholinoceptors (desensitization and postsynaptic potentiation) [7] and of the presynaptic autoreceptors [9], via K-channel blocking in the nerve terminal [2,8] or via changes in the activity of Na^+/K^+-ATPase, the catalytic subunit of which is structurally similar to the N-cholinoceptor [6]. The synaptic effect of AC may also be mediated by the increase in the intracellular K^+ concentration [4] due to activation of a large number of postsynaptic cholinoceptors. Since in our experiments the "aftereffect" of AC was entirely abrogated by the blockers of N- and M-cholinoceptors, being simulated by carbacholine but not being reproduced in the presence of the M-cholinoceptor agonist oxotremorin, the typical N- and M-cholinoceptors can hardly be the targets for AC in our case. The K^+ channels of the nerve terminal are also not likely to be such targets, since blocking of these channels with AC is not simulated by carbacholine and is not abrogated by N- and M-cholinoceptor blockers [2,8]. It is well known, however, that in a low concentration AC activates the membrane Na^+/K^+-ATPase and the muscle fiber electrogenic pump, and that such an effect of AC is abolished by N- and M-cholinoceptor blockers [5]. The observed muscle fiber hyperpolarization may be due to Na^+/K^+-ATPase activation in the presence of exogenous AC, and this enzyme system itself is the target (or one of the targets) mediating the "aftereffect" of AC in our experiments.

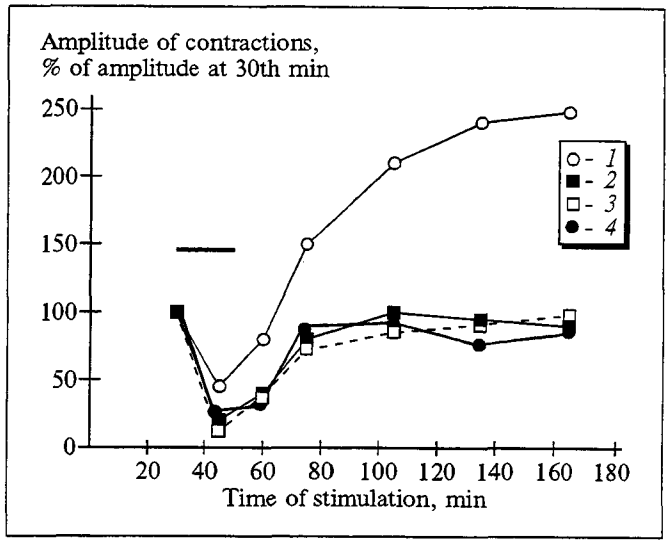

Fig. 2. Variation of amplitude of contractions of rat diaphragm during tetanization (nerve stimulation) in the presence of armine (*1*) and armine in combination with tubocurarine (*2*), scopolamine (*3*), and heparin (*4*) in the solution.

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