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Alkylating derivative of hexadecamethonium protects muscle synaptic acetylcholinesterase against inhibition

Anatoly F. Danilov [a], Victor M. Grigoriev [a,*], Natalya K. Prokhorenko [b], Oleg E. Sherstobitov [b]

[a] *Laboratory of Pharmacology, Sechenov Institute of Evolutionary Physiology and Biochemistry, Russian Academy of Sciences, St-Petersburg, Russian Federation*

[b] *Laboratory of Muscle Biochemistry, Sechenov Institute of Evolutionary Physiology and Biochemistry, Russian Academy of Sciences, St-Petersburg, Russian Federation*

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Abstract

The action of the alkylating derivative of hexadecamethonium on frog neuromuscular transmission was studied with the help of intracellular microelectrodes. Treatment of frog m. cutaneous pectoris-n. pectoralis preparations with the alkylating derivative of hexadecamethonium (0.5 μM) for 30 min led to an irreversible decrease in the amplitude of the end-plate potentials by 2.5-fold without a change of their latency period or quantal content. Such treatment led also to a considerable reduction of the anticholinesterase effects of neostigmine and of the organophosphorus irreversible inhibitor, armine. Thus, when applied to intact nerve-muscle preparations, neostigmine (2 μM) or armine (1 μM) increased the amplitude of end-plate potentials by 80–90%, and the rise time and half-decay time by about 2- to 3-fold. However, after the nerve-muscle preparations were pretreated with the alkylating derivative of hexadecamethonium (0.5 μM, for 30 min), the amplitude of end-plate potentials increased by 20–25%, rise time by 15–20% and half-decay time by 40–50% only. Investigation of muscle acetylcholinesterase activity, using the Ellman technique, showed that the alkylating derivative of hexadecamethonium diminished the sensitivity of the muscle acetylcholinesterase to inhibition without exerting its own inhibitory action.

Keywords: End-plate potential; Alkylating derivative of hexadecamethonium; Acetylcholinesterase inhibitor

1. Introduction

Irreversible blocking agents of acetylcholine receptors are used sometimes in the study of synaptic transmission of the cholinergic system. The first to be mentioned among them are α-neurotoxins of snake venoms, in particular, α-bungarotoxin, which has high affinity and specificity for acetylcholine receptors. Application of α-neurotoxins made it possible to solve many problems in neurophysiology and neuropharmacology (Magazanik, 1977; Chang, 1979). In some cases application of only one type of irreversible blocking agent can yield unreliable data, and therefore an attempt should be made to search for a new irreversible blocking agent.

Alkylating agents in a series of 2-halogenoethylamines are of special interest in this respect (Gill and Rang, 1966). Among them there are agents effective at both muscarinic and nicotinic receptors (Vulfius, 1975; Shelkovnicov and Michelson, 1977; Michelson and Shelkovnikov, 1980). Alkylating derivatives of decamethonium and hexadecamethonium have the highest potency as specific blocking agents of the acetylcholine receptors of skeletal muscles (Rang and Ritter, 1969; Michelson and Shelkovnikov, 1980). The technique used by the authors allowed them to estimate the action of alkylating agents on the postsynaptic membrane only. In the present work intracellular electrodes were used, which made it also possible to study the influence of an alkylating derivative of hexadecamethonium (hexadecamethylene-bis-methylchloroethylamine) on each stage of synaptic transmission.

* Corresponding author. Laboratory of Pharmacology, Sechenov Institute of Evolutionary Physiology and Biochemistry, Russian Academy of Sciences, pr. Thoreza 44, St-Petersburg 194223, Russian Federation. Tel. (812) 552-99-32, fax (812) 552-30-12.

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2. Materials and methods

2.1. Animals and solutions

All experiments were performed on frog (*Rana temporaria*) m. cutaneous pectoris-n. pectoralis preparations. The preparation was placed in an experimental chamber and superfused continuously with modified Ringer solution at 2.0–2.5 ml/min. The calcium concentration of the Ringer solution was decreased to 0.6 mM and the magnesium concentration was increased to 2.0 mM, to prevent muscle contractions during nerve stimulation. The temperature of the Ringer solution was 19–21°C; the pH was 7.2–7.4.

2.2. Intracellular recording of the end-plate potentials (e.p.p.)

e.p.p. were recorded with glass intracellular electrodes according to standard techniques (Fatt and Katz, 1951). Capillary microelectrodes filled with 2.5 M KCl had a resistance of about 15–20 MΩ. The nerve was stimulated with a suction electrode, 0.5/s frequency. The measurement of e.p.p. amplitudes and calculation of their average size were done automatically, as described earlier (Skliarov and Danilov, 1977; Skliarov, 1980). The quantal content of e.p.p. was determined from the coefficient of variation of the e.p.p. amplitudes (Del Castillo and Katz, 1954). It was not necessary to make a correction for non-linear summation because the e.p.p. amplitudes did not exceed 10 mV (McLachlan and Martin, 1981). At least 300 e.p.p. were used to calculate the quantal content. The latency period, rise time and half-decay time of e.p.p. were measured from recordings on photographic film.

2.3. Drugs

Alkylating agents containing the 2-chloroethylamine group are known to be cyclized in aqueous solution to the active ethylenimmonium ion (Fig. 1) which is capable of making a covalent bond with a receptor (Gill and Rang, 1966). At room temperature (20–22°C) and pH 7.4 the concentration of the ethylenimmonium ions reaches a maximum for about 1 h, which is equal, on average, to 20–30% of the initial concentration of the diluted agent and remains stable for several hours. Therefore the experiments were started 1 h after the agent was dissolved.

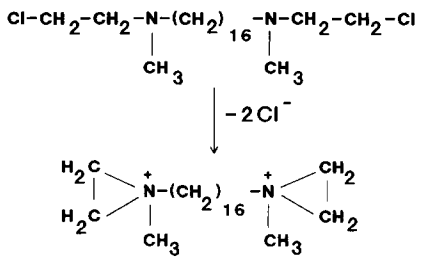

Fig. 1. Cyclization of the alkylating derivative of hexadecamethonium.

Neostigmine and the irreversible organophosphorus inhibitor, armine (USSR; Aluf, 1955), were used to inhibit muscle acetylcholinesterase.

The compounds were applied to neuromuscular preparations through the superfusion system.

2.4. Measurement of acetylcholinesterase activity

The action of the alkylating derivative of hexadecamethonium on acetylcholinesterase activity was estimated by using strips of m. cutaneous pectoris 2–3 mm wide, cut out from the muscle part located along the nerve. The strips were placed in test tubes filled with Ringer solution to which $MgCl_2$ 2.0 mM was added. Each sample consisted of 5 muscle strips. Acetylcholinesterase activity was determined according to the Ellman technique (Ellman et al., 1961), using acetylthiocholine bromide (1 mM) as a substrate. The rate of its hydrolysis was measured by spectrophotometry by measuring the extent of light absorption (wavelength 400 nm).

The antiacetylcholinesterase potency of irreversible inhibitors is expressed as a constant of the rate of the bimolecular reaction for enzyme inhibition (K_a; Aldridge and Reiner, 1969).

The experiments were carried out as follows. First, the initial activity of muscle acetylcholinesterase was determined in each group of strips. One group of strips served as control for repeated measurement of acetylcholinesterase activity. At the end of the experiment these strips were used to determine the concentration of sulfhydryl groups which had no relation to acetylcholinesterase activity. A second group of muscle strips was treated with the alkylating derivative of hexadecamethonium (10 μM) and then with armine (1.0–2.0 μM) for 3 min. After the measurement of acetylcholinesterase activity the strips were removed from the test tubes, washed and placed into other test tubes. A third group of strips was treated with armine at the above concentration for 3 min.

2.5. Statistical analysis

Statistical analysis was performed by means of Student's *t*-test for paired and unpaired observations. The results are presented as mean values ± S.E.M.

3. Results

3.1. Physiological study

Application of the alkylating derivative of hexadecamethonium to a nerve-muscle preparation resulted in a slow, gradual decrease of the e.p.p. amplitude with no alteration of the membrane potential of the muscle fibre. The e.p.p. amplitude continued to decrease in the presence of the agent in the solution (Fig. 2); the decrease was dose and time dependent. A 30-min application of the alkylating derivative of hexadecamethonium (0.5 μM) was followed by a 4-fold decrease of the amplitude accompanied by a small shortening of both fronts of the e.p.p. (Table 1). Wash-out of the preparation led to a gradual increase of the e.p.p. amplitude, which 40–60 min later reached a new stable level. The plateau was approximately 2.5-fold lower than the control level. At the same time there was a partial restoration of the time course of both fronts of the e.p.p. (Table 1). The alkylating derivative of hexadecamethonium had no influence on presynaptic events. First, it did not change the latency period of the e.p.p. Second, no change in the quantal content of the e.p.p. was revealed by computation. The partial restoration of the e.p.p. amplitude after wash-out showed that only some of the agent molecules make covalent bonds with acetylcholine receptors; the other molecules interact with the receptors as reversible ammonium compounds.

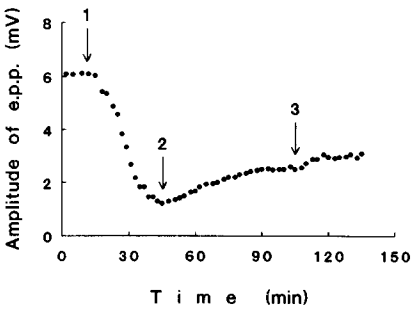

Fig. 2. Effects of the alkylating derivative of hexadecamethonium and neostigmine on the amplitude of the e.p.p. Abscissa: time in minutes; ordinate: amplitude of e.p.p. in mV. Replacement of solution is marked by arrows; (1) Ringer solution containing the alkylating derivative of hexadecamethonium (0.5 μM); (2) Ringer solution; (3) Ringer solution with neostigmine (2.0 μM).

It is known that inhibition of synaptic acetylcholinesterase prolongs the e.p.p. time course. In contrast, in our experiments, the alkylating derivative of hexadecamethonium caused a small shortening of both fronts of the e.p.p., which may be ascribed to diminution of the number of receptors able to interact with acetylcholine (Katz and Miledi, 1973). This suggested that the alkylating derivative of hexadecamethonium had no effect on synaptic acetylcholinesterase. However, pretreatment of the frog nerve-muscle preparations with the alkylating derivative of hexadecamethonium led to a considerable reduction of the effects of acetylcholinesterase inhibitors (neostigmine, armine). Thus, when applied to intact nerve-muscle

Table 1
Influence of the alkylating derivative of hexadecamethonium (0.5 μM) on e.p.p. parameters

	Control	Action for 30 min	Washing for 60 min
Amplitude (mV)	5.23 ± 0.46 (10)	1.11 ± 0.16 [a] (10)	2.11 ± 0.07 [a] (10)
Quantal content	39.4 ± 5.5 (8)	–	38.4 ± 6.2 (8)
Latency period (ms)	1.62 ± 0.14 (7)	1.59 ± 0.14 (7)	1.56 ± 0.13 (7)
Rise time (ms)	1.10 ± 0.08 (7)	0.76 ± 0.05 [a] (7)	0.91 ± 0.05 (7)
Half-decay time (ms)	2.55 ± 0.22 (7)	1.78 ± 0.14 [a] (7)	1.82 ± 0.16 [a] (7)

The data are presented as means ± S.E.M. The number of experiments is shown in brackets. [a] Significant difference ($P < 0.05$).

Table 2
The change in e.p.p. parameters under the influence of acetylcholinesterase inhibitors without and after pretreatment of nerve-muscle preparations with the alkylating derivative of hexadecamethonium (0.5 μM, 30 min)

	Neostigmine 2.0 μM		Armine 1.0 μM	
	Without	After	Without	After
Amplitude	1.81 ± 0.11 (8)	1.23 ± 0.06 [a] (6)	1.84 ± 0.05 (17)	1.22 ± 0.05 [a] (5)
Quantal content	1.17 ± 0.72 (4)	1.02 ± 0.06 (4)	0.99 ± 0.04 (12)	1.02 ± 0.07 (4)
Latency period	1.02 ± 0.01 (4)	1.01 ± 0.01 (5)	1.02 ± 0.02 (12)	1.00 ± 0.01 (4)
Rise time	1.58 ± 0.15 (4)	1.16 ± 0.06 [a] (5)	2.75 ± 0.18 (12)	1.15 ± 0.07 [a] (4)
Half-decay time	2.08 ± 0.07 (4)	1.35 ± 0.07 [a] (5)	3.14 ± 0.15 (12)	1.51 ± 0.25 [a] (4)

All data indicate a relative change in e.p.p. parameters after application of the acetylcholinesterase inhibitors. Initial values of the e.p.p. parameters were taken as 1. A mean of several experiments ± S.E.M. is shown. The number of experiments is given in brackets. [a] Results where pretreatment of nerve-muscle preparations led to a statistically significant difference ($P < 0.05$) in inhibitor action.

preparations, neostigmine (2 μM) or armine (1 μM) increased the e.p.p. amplitude by 80–90%, and the rise time and the half-decay time of the e.p.p. by about 2- to 3-fold. After the nerve-muscle preparations were pretreated with the alkylating derivative of hexadecamethonium (0.5 μM) for 30 min, the e.p.p. amplitude increased by 20–25%, the rise time by 15–20% and the half-decay time by 40–50% (Table 2, Fig. 2). Pretreatment of nerve-muscle preparations with the alkylating derivative of hexadecamethonium at higher concentrations (1–2 μM) led to a further reduction of the effects of acetylcholinesterase inhibitors: sometimes the latter gave no effect. This was found to have no relation with presynaptic events, because the latency period and the quantal content of the e.p.p. remained unchanged in the experiments (Table 2).

3.2. Biochemical study

As described in Materials and methods, the acetylcholinesterase activity of muscle strips was determined before and after incubation with the alkylating derivative of hexadecamethonium and armine. In the control group of muscle strips, acetylcholinesterase activity did not change during the entire experiment. Incubation of strips for 1 h with the alkylating derivative of hexadecamethonium (10 μM) did not lead to inhibition of acetylcholinesterase, but was followed by a significant decrease of the sensitivity of muscle acetylcholinesterase to armine. Thus, in the untreated muscle strips the K_a of armine was equal to $(2.65 \pm 0.07) \times 10^5$ M^{-1} min^{-1} ($n = 9$) while in the pretreated strips K_a was $(1.39 \pm 0.11) \times 10^5$ M^{-1} min^{-1} ($n = 8$), i.e. an almost 2-fold decrease in acetylcholinesterase sensitivity to armine. Insignificant inhibition of the muscle acetylcholinesterase was observed only after incubation of the muscle strips with the alkylating derivative of hexadecamethonium for 3 h or longer. Such treatment of muscle strips led to a further weakening of the anticholinesterase action of armine.

4. Discussion

The alkylating derivative of hexadecamethonium decreases irreversibly the sensitivity of the postsynaptic membrane to endogenous acetylcholine, and thus blocks neuromuscular transmission. However, it is of interest where the alkylating derivative of hexadecamethonium binds on the postsynaptic membrane.

If the blocking action of some compound can be prevented by a specific blocker capable of binding with the recognition site (active centre) of the acetylcholine receptor, then this compound binds to the recognition site. In experiments with skeletal muscles *d*-tubocurarine is usually used as a specific competitive neuromuscular blocking agent. It has been shown in experiments with muscle contractions that *d*-tubocurarine (50 μM) completely prevents the action of the alkylating derivative of decamethonium (Michelson and Shelkovnikov, 1980). In our experiments *d*-tubocurarine at the same concentration weakly diminished the effects of the alkylating derivative of hexadecamethonium. This means that the alkylating derivative of hexadecamethonium does not act only at the recognition site. As this agent does not block postsynaptic ion channels (Grigoriev et al., 1991), it may be suggested that the alkylating derivative of hexadecamethonium also binds to the allosteric site of receptors.

According to our data, the alkylating derivative of hexadecamethonium at the concentrations used had no effect on the activity of muscle acetylcholinesterase. However, pretreatment of the nerve-muscle preparations with the alkylating derivative of hexadecamethonium led to a considerable reduction of the effects of acetylcholinesterase inhibitors. This cannot be due to irreversible blocking of acetylcholine receptors by the alkylating derivative of hexadecamethonium, as it is known that blockade of acetylcholine receptors with irreversible blockers such as immunoglobulin G from patients with myasthenia gravis or α-bungarotoxin increases the influence of acetylcholinesterase inhibitors on the amplitude of miniature end-plate currents (e.p.c.). The change of the decay time constant of the miniature e.p.c. was near normal at the same time (Pennefather and Quastel, 1981). In our experiments with frog m. sartorius-n. ishiadicus preparations neostigmine (6.6 μM) increased the amplitude of the e.p.c. to 132% and the decay time constant to 390%. After an about 4-fold reduction of the e.p.c. amplitude caused by the alkylating derivative of hexadecamethonium, neostigmine at the same concentration increased the e.p.c. amplitude to 121% and the decay time constant to 129%. Under identical experimental conditions after the e.p.c. amplitude has been decreased by α-bungarotoxin to the same extent, neostigmine increased the e.p.c. amplitude to 167% and the e.p.c. time constant to 302% (Grigoriev et al., 1991).

As shown earlier, alkylating agents can irreversibly bind with acetylcholinesterase (Volkova and Kochetova, 1977, 1983). We therefore suggest that the alkylating derivative of hexadecamethonium interacts with muscle acetylcholinesterase and protects it against the action of inhibitors without affecting its activity per se.

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