INTERACTION BETWEEN MONOACETYLCOBROTOXIN-^{3}H AND MEMBRANE PREPARATIONS FROM DENERVATED MUSCLES AND THE CAUDATE NUCLEI

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KEY WORDS: monoacetylcobrotoxin; nicotinic cholinergic receptor; striated muscles; caudate nuclei of the brain; myasthenia.

Of all the brain structures the caudate nuclei are richest in acetylcholinesterase and choline-acetyltransferase — the enzymes of the cholinergic system. It was considered that nicotinic cholinergic receptors (NCR) are absent from these brain structures [1]. However, investigations indicating that NCR are present in these nuclei have recently been published [8]. The writers reported previously having isolated from a detergent extract of membranes from the caudate nuclei of the bovine brain a protein inducing myasthenia in rabbits, in the same way as the NCR-protein from the electric organ of fishes and from striated muscles [2]. Neurotoxins from venom of snakes belong to families of poisonous snakes and sea snakes, which block NCR of striated muscles and the electric organ of fishes highly specifically and firmly [4, 10-12], are widely used in the study of NCR. Meanwhile the use of neurotoxins for the investigation of NCR in nerve tissue has yielded contradictory results [6, 7, 15].

The object of this investigation was to compare NCR from striated muscles and from the caudate nuclei. The ^{3}H-monoacetyl derivative of the principal neurotoxin from the venom of the Central Asiatic cobra was used as marker for NCR.

EXPERIMENTAL METHOD

The NCR marker was obtained by acetylation of the principal neurotoxin (toxin P) of the venom of the Central Asiatic Cobra *(Naja naja oxiana)* [9, 12] by the method in [16], using a Soviet preparation of ^{3}H-acetic anhydride with a specific activity of 0.186 Ci/mmole. The acetyl derivatives of the neurotoxin were separated by the method of Karlson et al. [13]. Monoacetylocobrotoxin-^{3}H (MACT-^{3}H) with toxicity equal to that of the original neurotoxin was used (LD_{50} for albino mice was 0.1 μg/g). Membrane preparations were isolated from the caudate nuclei of cows' brains and from denervated rabbit gastrocnemius muscles (4 weeks after denervation). Denervation of the muscles was carried out to increase their content of NCR-protein [4]. A homogenate of caudate nuclei (10%) in 0.32 M sucrose solution was centrifuged at 1000g for 10 min; the resulting supernatant was centrifuged at 105,000g for 45 min. The fraction of plasma membranes was isolated from the muscle homogenate by the method of Boegman et al. [5]. Residues of membrane fragments isolated in both cases were washed with 0.01 M phosphate buffer, pH 7.6, with 0.1 M NaCl and kept in the cold until required for use. All operations for obtaining membrane preparations were carried out between 4 and 6°C. Binding of MACT-^{3}H with membrane fragments was studied by ultracentrifugation. Samples each of 100 mg membrane fragments in 0.4 ml of the above-mentioned buffer solution with NaCl were transferred to centrifuge tubes with a capacity of 10-12 ml (at room temperature) and 0.1 ml of the solution of the test substance or the same volume of buffer was added to each tube, followed by 0.1 ml of 10^{-5}M MACT-^{3}H solution of 30 min later. The samples were equilibrated 30 min later with cold buffer, the volume made up to 8-10 ml, and the samples were centrifuged at 105,000g for 45 min, after which the residue of membranes was washed twice more in the same way. The membranes were dissolved in 0.5 ml of a 10% solution of Triton X-100 with heating (60°C, 1 h), the suspension was added to scintillation solution, and radioactivity was measured on a model 3380 liquid scintillation counter (Packard, USA). The background level was determined beforehand for each flask with scintillator; this background value was subtracted from the radioactivity of the sample containing

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TABLE 1. Effect of Cobrotoxin, D-Tubocurarine, and Specific Rabbit Blood Serum on Binding of MACT-^{3}H (2×10^{-6}M) by Membrane Preparations

Preparation	Control	Cobrotoxin (10^{-5} M)	D-tubocurarine (10^{-4} M)	Serum (dilution 1:5)
Muscle membranes	156±15	5±2*	1+3*	106±22
Membranes of caudate nuclei	39±11	24±5	22±7	11±2*

Legend: 1) excess of radioactivity over background in cpm/100 g membranes. 2) Arithmetic mean of three samples and standard deviation. 3) Results differing significantly from control at the 99% level (by the U criterion [3]) marked by asterisk.

fragments of membranes. The counting time of each sample was 20-50 min. Blood serum was obtained from rabbits immunized with protein, isolated from the caudate nuclei of bovine brain [2].

EXPERIMENTAL RESULTS

Measurements of radioactivity during washing of the membranes showed that unbound MACT-^{3}H was eluted from the membranes of the denervated muscles during the first two washings, and that the third washing caused virtually no reduction in radioactivity in the membranes. The results of investigation of binding of MACT-^{3}H by membrane preparations (after triple washing) are given in Table 1. Binding of MACT-^{3}H by muscle membranes was blocked by high concentrations of D-tubocurarine (10^{-4}M) and cobrotoxin (10^{-5}M). The concentration of binding sites for MACT-^{3}H in the preparation of muscle plasma membranes was about 5×10^{-12} mole/mg protein, close to the concentration of NCR [4]. These results show that MACT-^{3}H blocks NCR of muscle membranes specifically and firmly, in the same way as neurotoxins of venom of snakes belonging to the cobra family [4, 10, 11].

By contrast with muscle membranes, membranes from the caudate nuclei bound MACT-^{3}H less firmly: Radioactivity remaining bound with the membrane after the second washing was reduced approximately by half on the next washing; both cobrotoxin and D-tubocurarine reduced binding of MACT-^{3}H approximately by 40% during both the second and the third washings (relative to the corresponding control).

Interaction between neurotoxins and NCR of striated muscles and the electric organ of fishes is known to take place in two stages: Initially a highly specific complex is formed, but after a short time this becomes extremely stable [11]. On the basis of these results it can be postulated that the formation of the stable complex of MACT-^{3}H with NCR of the caudate nuclei takes place with difficulty and interaction is limited to the first specific and largely reversible stage.

Blood serum taken from rabbits immunized with a protein preparation from the caudate nuclei [2] and exhibiting features of myasthenia reduced the binding of MACT-^{3}H by membranes of the caudate nuclei by a greater degree than muscle membranes (Table 1). The serum of rabbits in which no myasthenia was observed after immunization had a similar but weaker action. The severity of myasthenia in animals immunized with NCR-protein is known to depend on their serum level of antibodies capable of blocking NCR of the neuromuscular junction [14], in agreement with the results of the present investigation.

On the basis of this investigation several conclusions can be drawn regarding the properties of this cholinergic receptor of the caudate nuclei. First, its inclusion in the nicotinic type means that the membranes of the caudate nuclei can bind MACT-^{3}H — a specific marker of NCR. Second, this NCR differs considerably from the NCR of muscles, for binding of MACT-^{3}H by membranes of the caudate nuclei is much less stable. Third, the NCR of the caudate

nuclei and muscles are similar in their immunologic properties, whereas differences in the antigenic properties of these receptors are revealed by investigation of the effect of specific serum on binding of MACT-^{3}H by the membrane preparations studied. The results thus confirm the view that the syndrome of myasthenia described previously [2] is connected with an autoimmune process involving NCR of the neuromuscular junction, which is similar in its properties to NCR of the caudate nuclei.

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CENTRAL CHOLINOPOTENTIATING ACTION OF BENACTYZINE

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KEY WORDS: benactyzine; small doses; after-effect; cholinopotentiating action; arecoline.

A model of arecoline tremor and salivation has been suggested for the study of relations between the central and peripheral components of cholinolytic action [2]. This model later began to be widely used to study the mechanism of action of drugs and their mixtures [3, 9]. The comparative cholinolytic activity of various preparations is revealed as antagonism with cholinomimetics [10]. In these cases the cholinomimetic behaves as a factor causing disturbance of the system and the cholinolytic as a factor bringing it to rest. By means of this approach the cholinergic nature of brain systems can be revealed. Gromov [4] has used cholinomimetic testing after preliminary administration of benactyzine.

It is stated in the literature that cholinolytics have opposite effects on certain physiological reactions depending on their dose. Scopolamine, for example, increases aggression in rats in small doses, but in large doses inhibits it [13]. Scopolamine has a similar biphasic action on the behavior of rats in an "open field" [14].

In the present investigation the central action of benactyzine was investigated on a model of arecoline tremor over a wide range of doses and time intervals.

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