Biochemical Pharmacology, Vol. 16, pp. 1135–1138. Pergamon Press Ltd. 1967. Printed in Great Britain

Ribonucleoprotein in human skeletal muscle with affinity for *d*-tubocurarine and acetylcholine

(*Received* 26 *October* 1966; *accepted* 20 *January* 1967)

IN RECENT years attempts have been made to isolate and characterize the macromolecular components of excitable tissues which may play a role in excitation or conduction. These include an acidic mucopolysaccharide isolated from the electric organ of the electric eel,[1] a protein from the electric organ[2] or medullated nerve tissue of the dog and frog,[3] and a phosphatide isolated from various tissues including skeletal muscle.[4] The present study reports the properties of a ribonucleoprotein isolated from human skeletal muscle by precipitation with *d*-tubocurarine.

Human skeletal muscle was obtained by biopsy or at postmortem examination within 5 hr after death. Muscle was homogenized for 80 sec in a Waring blender with an equal weight of phosphate-buffered saline (0·15 M NaCl and 0·02 M sodium phosphate buffer, pH 7·3), and centrifuged at 13,300 *g* for 15 min. The supernatant was dialyzed against phosphate-buffered saline, centrifuged again, and the supernatant recovered as saline extract of muscle.

One vol. of 0·05 M *d*-tubocurarine chloride solution was mixed with 4 vol. of saline extract of muscle and, after 18 hr, the resulting precipitate was centrifuged at 13,300 *g* for 15 min. The precipitate was suspended in phosphate-buffered saline and dialyzed for 16 hr against phosphate-buffered saline to remove *d*-tubocurarine. Most of the precipitate dissolved, and the undissolved residue was removed by centrifugation at 13,300 *g* for 15 min. The supernatant was again processed as described above, except that the mixture with *d*-tubocurarine was allowed to stand for 5 hr before centrifugation, as the precipitate developed more rapidly. The product was an opalescent solution which became a white powder on lyophilization. All procedures were carried out at 4°.

The yield of isolated substance was 394 ± 72 μg/g of muscle (M ± S.E. in 15 experiments). Nitrogen (11·2 ± 0·9 per cent of dry weight, M ± S.E. in 6 experiments), carbon, hydrogen, sulfur, and phosphorus were present. The reactions for protein were positive, and amino acids were identified by chromatography of the hydrolysate. The orcinol reaction indicated the presence of pentose in acid hydrolysate, and adenine, guanine, cytosine, and uracil were found in molecular ratio of 14·0 ± 0·72/26·0 ± 1·18/38·1 ± 3·26/21·9 ± 3·47 (M ± S.E. in 6 experiments) after isolation by paper chromatography. Desoxypentose and thymine were absent. The findings indicate that the substance is a ribonucleoprotein. RNA per 100 g protein measured by the method of Schneider was 36·3 ± 5·35 g (M ± S.E. in 22 experiments), as compared to 0·735 ± 0·067 g (M ± S.E. in 18 experiments) in saline extract of muscle. Tropomyosin, actomyosin, phospholipid, and *d*-tubocurarine could not be identified. The ultraviolet and infrared absorption spectra were also compatible with nucleoprotein. In free-boundary electrophoresis, the substance separated into three fractions, which contained 89, 9 and 2 per cent of protein, with mobility of $2{\cdot}58 \times 10^{-5}$, $6{\cdot}35 \times 10^{-5}$, and $12{\cdot}65 \times 10^{-5}$ cm²/volt sec respectively. Fractionation on a DEAE-cellulose column yielded a major fraction, eluted by 0·65 M NaCl, and two minor fractions. Saline extract of muscle, on the other hand, was eluted for the most part by 0·15 M NaCl. Muscle ribonucleoprotein was not retained in a column of Sephadex G-200, and ultracentrifugation studies indicated that the sedimentation constant is about 9·6S and the molecular weight approximately 300,000.

Muscle ribonucleoprotein demonstrated a strong cholinesterase activity of 9·24 (m-mole acetylcholine bromide hydrolyzed/hr/mg protein, measured by the method of Hestrin), as compared with 1·83 in saline extract of muscle. The protein also hydrolyzed acetyl-β-methylcholine but not butyrylcholine or benzoylcholine. Aldolase activity was present, but was only about one eighth that of saline extract of muscle, per unit of protein. Adenosine triphosphatase and glucose 6-phosphatase activity could not be demonstrated.

Binding of ^{14}C-labeled dimethyl-*d*-tubocurarine or acetylcholine to muscle ribonucleoprotein was several times greater than to saline extract of muscle, per unit of protein (Table 1). When cholinesterase activity was inhibited by DFP, the binding of ^{14}C from acetyl-^{14}C-choline was reduced ($P < 0{\cdot}001$), while the binding of ^{14}C-dimethyl-*d*-tubocurarine was not altered. This suggests that

^{14}C-dimethyl-*d*-tubocurarine bound to noncholinesterase sites, and that ^{14}C from acetyl-^{14}C-choline bound to both cholinesterase and noncholinesterase sites. Equimolar concentrations of acetylcholine reduced binding of ^{14}C-dimethyl-*d*-tubocurarine, and equimolar concentrations of *d*-tubocurarine reduced binding of ^{14}C from acetyl-^{14}C-choline, with or without DFP.

TABLE 1. BINDING OF ^{14}C-DIMETHYL-*d*-TUBOCURARINE AND ACETYL-^{14}C-CHOLINE TO RIBONUCLEOPROTEIN AND SALINE EXTRACT OF HUMAN SKELETAL MUSCLE

	Bound compound* (μmoles/mg protein)			
	^{14}C-Dimethyl-*d*-tubocurarine		Acetyl-^{14}C-choline	
	To ribonucleoprotein	To saline extract	To ribonucleoprotein	To saline extract
Control	0·680 ± 0·019	0·218 ± 0·021	4·492 ± 0·023	1·210 ± 0·020
Plus:				
10 μM Acetylcholine	0·595 ± 0·018 ($P < 0·02$)	0·188 ± 0·009		
10 μM *d*-Tubocurarine			4·251 ± 0·022 ($P < 0·001$)	0·823 ± 0·019 ($P < 0·001$)
10 μM DFP	0·623 ± 0·028	0·209 ± 0·004	3·168 ± 0·045 ($P < 0·001$)	0·363 ± 0·016 ($P < 0·001$)
10 μM DFP + μM acetylcholine	0·540 ± 0·017 ($P < 0·05$)	0·180 ± 0·004 ($P < 0·001$)		
10 μM DFP + μM *d*-tubocurarine			2·913 ± 0·049 ($P < 0·001$)	0·295 ± 0·114

* Measured by assay of ^{14}C of samples obtained from equilibrium dialysis of 1 ml sample (4 mg protein) against 6 ml 10 μM ^{14}C-labeled compounds in 0·15 M NaCl with 0·02 M sodium phosphate buffer. The Donnan effect was negligible. Values are mean of 6 experiments ± S.E. P values indicate significance of the reduction of binding compared to the control value (2nd to 4th lines), or to the control plus 10 μM DFP (last 2 lines).

Muscle ribonucleoprotein was precipitated from solution by addition of *d*-tubocurarine at concentrations of 0·05 mM or higher, or of dimethyl-*d*-tubocurarine at concentrations of 4 mM or higher. This precipitation was inhibited or partially reversed by acetylcholine and choline, and to a lesser extent by decamethonium and succinylcholine at concentrations 5 or more times higher than the concentration of *d*-tubocurarine. The other neuromuscular compounds studied had no effect except for procaine or urea which prevented the precipitation by *d*-tubocurarine at concentrations of 1 M or higher.

There was no difference in properties of muscle ribonucleoprotein isolated from muscle of patients with myasthenia gravis and individuals with no neuromuscular disorders.

The minimal concentration of *d*-tubocurarine that precipitated muscle ribonucleoprotein, 0·05 mM, is somewhat greater than the concentration of *d*-tubocurarine necessary to paralyze the isolated rat diaphragm, 1 μM,[5] and the plasma level necessary to produce respiratory paralysis in man, 5 μM.[6] Manyfold higher concentrations of antagonists were necessary to inhibit or reverse the precipitation, and there was only partial competitive binding in equilibrium dialysis between equimolar concentrations of dimethyl-*d*-tubocurarine and acetylcholine. A similar excess of these antagonists is required in order to partially reverse neuromuscular block due to *d*-tubocurarine,[7] and a large dose of neostigmine does not prevent the binding of ^{14}C-labeled curarine to the motor endplate of mouse diaphragm.[8] The dissociation constant of the reaction between ^{14}C-dimethyl-*d*-tubocurarine and muscle ribonucleoprotein was approximately 3×10^{-5} M. The apparent dissociation constant *in vivo* in the electric organ of the electric eel has been reported to be $2·4 \times 10^{-7}$ M in the reaction between

d-tubocurarine and the receptor, and $4{\cdot}4 \times 10^{-5}$ M in the reaction between carbamylcholine and the receptor.[9]

If we consider the yield and the molecular weight of the protein, and assume that each gram of muscle contained 10^5 fibers, at least 8×10^9 molecules of muscle ribonucleoprotein are present in each muscle fiber. This figure is higher than the number of molecules of acetylcholine or *d*-tubocurarine reported to react with each motor endplate. When applied by a microjet, $3{\cdot}3 \times 10^8$ acetylcholine molecules were required to excite a single endplate of frog muscle.[10] After stimulation by a single impulse, $3{\cdot}3 \times 10^5$ acetylcholine molecules per endplate were recovered from mouse diaphragm.[11] A single motor endplate has been reported to bind with $2{\cdot}8 \times 10^6$ molecules of curarine or $8{\cdot}2 \times 10^6$ molecules of decamethonium.[8] On the other hand, the motor endplate is said to be fully inhibited when less than 2 per cent of the total surface area is covered by curarine.[8] The receptor substance is postulated to be present in an inactive state throughout the muscle fiber, since denervation of muscle is followed by the spread of acetylcholine reactive area from the endplate region to the entire muscle fiber.[12] Immunologic studies are of interest in this regard, as the serum globulin of rabbits immunized with muscle ribonucleoprotein was bound with cross striations throughout the muscle fiber, indicating the presence of this substance or of a substance with similar antigenic determinants throughout the muscle.[13]

Since a ribonucleoprotein molecule is a polyanion, having many phosphate groups, these anionic groups are likely to be the sites that react with *d*-tubocurarine or acetylcholine. The reacting force between the neuromuscular compounds and the receptor has been a matter of speculation, but the phosphate group of the receptor is considered to play an important role.[14] The anionic sites of acetylcholinesterase have been postulated to be the receptor sites.[15]

Although muscle ribonucleoprotein isolated in the present study does not seem to be the acetylcholine receptor substance, further studies are indicated in view of the interesting properties of the ribonucleoprotein.

Acknowledgments—The authors are indebted to Mr. Panton H. Ebanks, Mr. Henry H. Broer, and Miss Barbara F. Matarese for their technical assistance, and to Dr. Abraham R. Kantrowitz for his cooperation. This study was supported by grants from the National Institutes of Health (NB 03464) and the Health Research Council of the City of New York (U-1025).

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Biochemical Pharmacology, Vol. 16, pp. 1138–1140. Pergamon Press Ltd. 1967. Printed in Great Britain

Return of γ-aminobutyrate transaminase activity in mouse brain after inhibition by aminooxyacetic acid: Chemical and histochemical observations*

(*Received* 4 *November* 1966; *accepted* 21 *December* 1966)

VIRTUALLY all the data in the literature are consistent with the interpretation that the steady-state levels of γ-aminobutyric acid (γABA) in the central nervous system (CNS) *normally* are governed by the glutamic acid decarboxylase activity (GAD) and not by the γABA-α-ketoglutarate transaminase activity (γABA-T).[1] It was shown upon administration to animals of hydroxylamine or aminooxyacetic acid (AOAA), substances which are potent inhibitors of both the GAD and γABA-T *in vitro*, that only the γABA-T was inhibited and that there were marked elevations of γABA content in the brains of the treated animals.[2] This suggested that the first two enzymes of the "γABA shunt" are not present in the same location in the CNS. Studies with cell-fractionation procedures have given results consistent with the interpretation that γABA-T is strictly a mitochondrial enzyme found largely at postsynaptic sites and that at least 40–50 per cent of the GAD activity is located in presynaptic nerve endings.[3–5] Much of the available data could be explained if it were assumed that γABA is a presynaptically liberated inhibitory transmitter by some neurons in various areas of the CNS and that after transport into postsynaptic neuronal sites it is metabolized in mitochondria by a sequence of reactions beginning with γABA-T. A histochemical procedure has been developed for the visualization of the γABA-T-succinic semialdehyde dehydrogenase sequence (γABA-T-S), the endpoint of which is the deposition of an insoluble formazan derivative from a tetrazolium salt (NitroBT), presumably at or close to the site of γABA-T activity.[6, 7] The purpose of the present study was to attempt to correlate the enzymatically measured values for γABA-T with the visually observed formazan deposition in sections of mouse brain, with a view to ascertaining whether or not the histochemical procedure has validity as a semiquantitative monitoring method for γABA-T activity in various loci in the CNS. The depletion and repletion of γABA-T activity in mouse brain after i.p. administration of AOAA was followed by both methods.

Fasted Swiss mice of both sexes, 22–25 g in weight, were injected i.p. with a neutral solution of AOAA (25 mg/kg), and animals were sacrificed by cervical dislocation at various times after the injection. The brains were removed quickly and frozen in dry ice. Ten 10-mμ sections were cut in a cryostat through the cerebrum at the same level in each brain and were placed in 1·0 ml of cold water. The next five sections were placed on slides for histochemical study. Another ten sections of

* This investigation was supported in part by Grant B-1615 from the National Institute of Neurological Diseases and Blindness and by a grant from the National Association of Mental Health, New York, N.Y.