in details from that of the vertebrates. However, the ground plan in the two groups is similar, for octopine and octopine dehydrogenase are formally analagous to lactate and lactate dehydrogenase in the vertebrates, both enzymes serving to sustain an oxidizing potential during anaerobic glycolysis.

P. W. HOCHACHKA*
P. H. HARTLINE†
J. H. A. FIELDS‡

Bindoy Village, Negros Oriental, The Philippine Republic

References and Notes

1. P. W. Hochachka, T. W. Moon, T. Mustafa, K. B. Storey, *Comp. Biochem. Physiol. B* **52**, 151 (1975).
2. J. H. A. Fields and P. W. Hochachka, *ibid.*, p. 158; F. Regnouf and N. van Thoai, *ibid.* **32**, 411 (1970); N. van Thoai, C. Huc, D. B. Pho, A. Olomucki, *Biochim. Biophys. Acta* **191**, 46 (1969).
3. B. Sacktor, *Adv. Insect Physiol.* **7**, 267 (1970); *Biochem. Soc. Symp.* **41**, 111 (1976); P. W. Hochachka, *ibid.*, p. 3.
4. K. B. Storey and P. W. Hochachka, *Comp. Biochem. Physiol. B* **52**, 169 (1975); *ibid.*, p. 175.
5. G. Gäde, personal communication at the July 1975 British Biochemical Society Symposium.
6. The procedure for isolating octopine dehydrogenase was as follows. Animals that were freshly captured in baited traps were removed from their shells, and the desired muscle was quickly excised, then homogenized usually in five or ten parts of 100 m*M* imidazole buffer (*p*H 7.0). The homogenate was spun in an RC-2B Sorvall refrigerated centrifuge at 4°C and 12,000*g* for 20 minutes. The supernatant solution contained essentially all extractable octopine dehydrogenase. For studies of its kinetic properties the enzyme was partially purified and prepared as described by Fields *et al.* (*10*). The enzyme was assayed in either a Unicam SP 1800 recording spectrophotometer or a Beckman DB recording instrument by following the change in optical density at 340 nm due to either NADH oxidation (in the forward direction) or NAD^+ reduction (in the back reaction). Assay conditions for the forward reaction: 0.2 m*M* NADH, varying pyruvate or arginine concentrations, 100 m*M* imidazole buffer (*p*H 7.0) at 25°C and a pressure of 1 atm; for the back reaction: 1.0 m*M* NAD^+, varying octopine concentrations, and 100 m*M* tris-HCl buffer at varying *p*H.
7. P. W. Hochachka and J. Meredith, in preparation.
8. K. Johansen, personal communication of observations made during the same nautilus expedition.
9. P. W. Hochachka, in preparation.
10. N. van Thoai, C. Huc, D. B. Pho, A. Olomucki, *Biochim. Biophys. Acta* **191**, 46 (1969); G. Gäde and M. Grieshaber, *J. Comp. Physiol.* **102**, 149 (1975); J. H. A. Fields, J. Baldwin, P. W. Hochachka, *Can. J. Zool.* **54**, 871 (1976).
11. A partially purified preparation of *Nautilus* ODH (free of contaminating enzymes such as lactate dehydrogenase, malate dehydrogenase, α-glycerophosphate dehydrogenase, isocitrate dehydrogenase, malic enzyme, or glucose 6-phosphate dehydrogenase) was used to assay octopine concentrations by procedures described by J. R. Williamson and B. E. Corky [*Methods Enzymol.* **13**, 434 (1969)]. Pyruvate levels in perchloric acid extracts of rapidly frozen tissues were assayed with the use of purified lactate dehydrogenase (Sigma). Arginine concentrations were determined on an amino analyzer by standard procedures.
12. Muscle preparations, either dissected cirrus muscle from the spadix or strips of retractor muscle, were hung vertically at room temperature (24°C) attached to a constant 50-g weight as a work load. The muscles were kept in seawater during most manipulations but stimulation always occurred in air. Stimulus conditions: 150 volts, 10-msec direct electrical stimulation of muscle at 10 hertz, for time periods described in the figure lengends. One 150-volt, 10-msec biphasic stimulus pulse was more than twice the intensity that evoked maximal twitch amplitude in a rested muscle strip. A 10-hertz stimulation caused smooth tetanic contraction of the muscle to between 66 and 50 percent of its relaxed length under 50-g loading. A continuously tetanized muscle relaxed to within 5 percent of its original length in about 15 minutes, and thereafter could produce no further work against the 50-g weight even after a 10-minute rest period.
13. The R.V. *Alpha Helix* expedition was funded by the NSF. P.W.H. was supported by an operating grant from the NRC (Canada); J.H.A.F. was an NRC predoctoral scholar; P.H.H. was supported by NIH (EY 01539). Especial thanks are due Dr. Jim Redmond for inviting us on his nautilus expedition and to Wilson Vailoces and his family and friends for introducing us to the haunts of the *Nautilus*. Dr. John Arnold introduced us to the spadix.

* Permanent address: Department of Zoology, University of British Columbia, Vancouver, B.C., Canada V6T 1W5.
† Permanent address: Department of Physiology, University of Illinois, Urbana 61801.
‡ Permanent address: Department of Zoology, University of Washington, Seattle 98105.

9 June 1976; revised 21 July 1976

Thymic Muscle Cells Bear Acetylcholine Receptors: Possible Relation to Myasthenia Gravis

Abstract. *Culture of dissociated thymus from rats and humans yielded cells identical to skeletal muscle with respect to morphology, contractility, electrophysiological properties, and the presence of acetylcholine receptors. These cells, strategically located in the thymus, may play a role in initiation of the autoimmune response against acetylcholine receptors, which is characteristic of myasthenia gravis.*

The thymus gland has long been suspected of playing an important role in myasthenia gravis (MG), a disorder manifested by weakness and fatigability of muscles. Clinical pathological evidence implicating the thymus in MG includes a high incidence of thymic hyperplasia (approximately 65 percent) and neoplasia (approximately 10 percent) in patients with MG (*1*), and beneficial effects of thymectomy in many patients (*2*). Studies of acetylcholine receptors (AChR) provide further evidence for a possible link between the thymus and MG. At present, the basic abnormality in MG is generally thought to be a reduction of available AChR at neuromuscular junctions (*3*), resulting from an autoimmune attack directed against receptors (*4*, *5*). Extracts of thymic tissue have now been shown to contain AChR (*5*, *6*), suggesting that an autoimmune reaction against AChR might be initiated within the thymus gland itself.

The source of AChR in the thymus is not yet known. In our study, we have considered the possibility that the AChR might be situated on "myoid" or musclelike cells, first described in the thymus in 1905 (*7*). Such cells are difficult to study in situ, because they are embedded within the tissue mass of the thymus gland. We have therefore used tissue culture techniques (*8*) to obtain sufficient quantities of intact cells for characterization of certain of their physiological, pharmacological, and morphological properties. The results indicate that muscle cultured from human or rat thymus is a rich source of AChR; further, thymic muscle corresponds to cultured skeletal muscle derived from more conventional sites of origin in all properties tested.

Thymuses were dissected from 8-week-old Sprague-Dawley rats, with care being taken to ensure that no tissue from the adjacent areas was included. For each experiment six thymuses were minced, trypsinized, and mechanically dissociated. The thymic cells were plated on plastic cover slips or in plastic wells and maintained in Dulbecco's modified Eagle's medium (*9*) supplemented

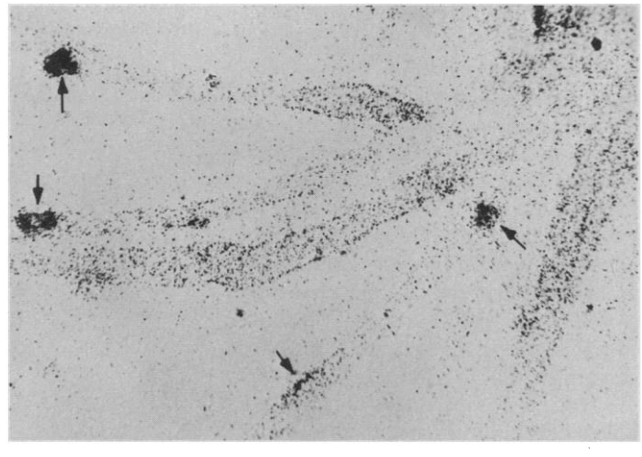

Fig. 1. Autoradiogram of rat thymic myotubes in cell culture after incubation with ^{125}I-labeled α-bungarotoxin. Grains are present over myotubes, an indication of surface acetylcholine receptors. The arrows mark hot spots, or areas of high AChR concentration.

with 10 percent fetal calf serum. After 24 hours, the unattached cells were washed away. The attached cells continued to proliferate until a monolayer was formed. In 3 to 5 days, spindle-shaped myoblastlike cells began to line up and fuse into multinucleated cells, identical in appearance to myotubes cultured from rat skeletal muscles (*10*). Other cells in the cultures appeared to be mainly fibroblasts. Myotubes also grew in abundance from a human thymus removed from a myasthenic patient and cultured in the same way. By 10 to 14 days, cross striations and spontaneous contractions were observed and recorded with microcinematography. Contraction could also be elicited by electrical stimulation with square pulses of 1-msec duration and 5-volt amplitude across two electrodes in the medium.

^{125}I-labeled α-bungarotoxin (α-BuTx) was used to detect the presence of AChR in the cultured cells by autoradiographic and radiometric methods (*11*); α-BuTx has been shown to bind specifically, and essentially irreversibly, to AChR (*12*), and thus has been used as a marker for AChR sites (*13*). The number of α-BuTx binding sites is proportional (probably equal) to the number of AChR sites, and the terms are therefore used interchangeably in this report. The rat thymus cultures were incubated with ^{125}I-labeled αBuTx for 30 minutes, and then thoroughly washed. For autoradiography, the cultures on the cover slips were fixed with 2 percent glutaraldehyde, dehydrated, and dried in air. They were then coated with Kodak NTB-2 emulsion and developed after exposure for 3 to 6 days. Silver grains were observed over all of the multinucleated myotubes, but not over other types of cells (Fig. 1). One noticeable feature was the distinct areas of high receptor concentration, or "hot spots," similar to those reported for cultured skeletal muscle (*14*). The grain density over the hot spots was 10 to 20 times higher than that over the rest of the myotube membrane.

In some cultures we also made quantitive measurements of receptors (*11*). The cultures were incubated with ^{125}I-labeled α-BuTx and then washed. The receptors were solubilized with 1 percent Triton, and the radioactivity was counted in a gamma spectrometer with a counting efficiency of 82 percent. Each 16-mm culture well contained $(1.82 \pm 0.27) \times 10^{10}$ receptors (S.E.M.), similar to the values for rat skeletal muscle cultures of comparable age and cell density (*15*).

To determine the ACh sensitivity of these cells, ACh was applied by conventional microiontophoretic methods, while membrane potentials were monitored with intracellular microelectrodes. The resting membrane potentials of the thymic myotubes after 15 days in culture ranged from -40 to -60 mv, the mean being -47 ± 3.75 mv (S.E.M.), and spontaneous bursts of action potentials were often observed. The ACh sensitivity of these muscle cells ranged from 10 to 60 mv per nanocoulomb, the mean being 24 ± 9.41 (Fig. 2). These findings demonstrate the presence of physiologically functional AChR's on the thymus-derived cells; the results are closely comparable to those in typical cultured skeletal muscle cells derived from rat limb muscles (*16*).

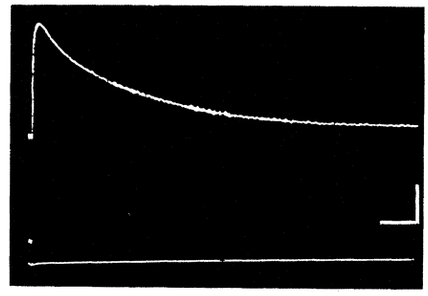

Fig. 2. Response of thymic myotube in culture to iontophoretically applied ACh. Upper trace: Intracellular recording of membrane potential. Lower trace: ACh pulse of 2-msec duration and 1.5-μa amplitude. Calibration: 5 mv, 10 msec. The ACh sensitivity is calculated to be 56 mv per nanocoulomb.

In order to ascertain whether the thymic cells were capable of forming neuromuscular contacts we cocultured them with cholinergic neuronal cells of a recently described (*17*) stable line (NG 108-15). Contacts developed between processes of the neuronal cells and the thymic cells, similar in appearance to those described for NG 108-15 cells and skeletal muscle (*17*).

Our findings suggest that cells cultured from rat and human thymus are identical to muscle cells derived from more conventional sources: they are striated and multinucleated, contract spontaneously and in response to electrical stimulation, have appropriate resting membrane potentials and ACh sensitivities, and appear to form contacts with cholinergic neuronal cells. Perhaps most important is the finding of AChR's associated with these cells. On the basis of the identity of these properties, it seems reasonable to consider the thymus-derived cells to be true skeletal muscle.

Because of their strategic location within the thymus, these receptor-bearing muscle cells may be vulnerable to immune attack. Some alteration in either the muscle cells or the immunocompetent lymphocytes of the thymus (*18*) may serve to break tolerance, and thereby initiate an autoimmune response directed against AChR as well as other components of skeletal muscle. Such a process would fit well with present concepts (*19*) of the pathogenesis of MG as an autoimmune disorder directed primarily against AChR.

ING KAO
DANIEL B. DRACHMAN
Department of Neurology,
Johns Hopkins Medical School,
Baltimore, Maryland 21205

References and Notes

1. H. Oppenheim, *Die Myasthenische Paralyse* (Karger, Berlin, 1901), p. 121; C. Weigert, *Neurol. Centralbl.* **20**, 597 (1901); C. Hart, *Virchows Arch. Pathol. Anat.* **220**, 185 (1915); E. T. Bell, *J. Nerv. Ment. Dis.* **45**, 130 (1917); B. Castelman and E. H. Norris, *Medicine* **29**, 27 (1949).
2. A. Blalock, M. F. Mason, H. J. Morgan, S. S. Riven, *Ann. Surg.* **110**, 554 (1939); G. Keynes, *Br. Med. J.* **2**, 611 (1949); J. A. Simpson, *Brain* **81**, 112 (1958); V. P. Perlo, B. Arnason, D. Poskanzer, R. S. Schwab, K. E. Osserman, A. Papatestas, L. Alpert, A. Kark, *Ann. N.Y. Acad. Sci.* **183**, 308 (1971); G. Genkins, A. E. Papatestas, S. H. Horowitz, P. Kornfield, *Am. J. Med.* **58**, 517 (1975).
3. D. M. Fambrough, D. B. Drachman, S. Satyamurti, *Science* **182**, 293 (1973).
4. J. Patrick and J. Lindstrom, *ibid.* **180**, 871 (1973); R. R. Almon, C. G. Andrew, S. H. Appel, *ibid.* **186**, 55 (1974); K. V. Toyka, D. B. Drachman, A. Pestronk, I. Kao, *ibid.* **190**, 397 (1975).
5. J. M. Lindstrom, V. A. Lennon, M. E. Seybold, S. Whittingham, *Ann. N.Y. Acad. Sci.* **274**, 254 (1976).
6. A. Aharonov, B. Tarrab-Hazdai, O. Abramsky, S. Fuchs, *Proc. Natl. Acad. Sci. U.S.A.* **72**, 1456 (1975).
7. J. A. Hammer, *Anat. Anz.* **27**, 41 (1905).
8. I. Törő, L. Gazsó, R. O. Török, I. Oláh, *Z. Zellforsch.* **89**, 241 (1968); H. Wekerle, B. Paterson, U.-P. Ketelsen, M. Feldman, *Nature (London)* **256**, 493 (1975).
9. R. Dulbecco and G. Freeman, *Virology* **8**, 396 (1959).
10. D. Yaffe and S. Fuchs, *Dev. Biol.* **15**, 33 (1967).
11. D. M. Fambrough, *J. Gen. Physiol.* **64**, 468 (1974).
12. D. K. Berg, R. B. Kelly, P. B. Sargent, P. Williamson, Z. W. Hall, *Proc. Natl. Acad. Sci. U.S.A.* **19**, 147 (1972).
13. P. N. Devreotes and D. M. Fambrough, *J. Cell Biol.* **65**, 335 (1975).
14. A. J. Sytkowski, Z. Vogel, M. W. Nirenberg, *Proc. Natl. Acad. Sci. U.S.A.* **70**, 270 (1973).
15. I. Kao, unpublished observations.
16. D. M. Fambrough and J. E. Rash, *Dev. Biol.* **26**, 55 (1971).
17. P. Nelson, C. Christian, M. Nirenberg, *Proc. Natl. Acad. Sci. U.S.A.* **73**, 123 (1976).
18. J. D. Smiley, J. Bradley, D. Daly, M. Ziff, *Clin. Exp. Immunol.* **4**, 387 (1969); R. M. Armstrong, R. M. Nowak, R. E. Falk, *Neurology* **23**, 1078 (1973); N. I. Abdou, R. P. Lisak, B. Zweiman, I. Abrahamsohn, A. S. Penn., *N. Engl. J. Med.* **291**, 1271 (1974).
19. G. Goldstein and W. W. Hofmann, *J. Neurol. Neurosurg. Psychiatry* **31**, 453 (1968); S. K. Datta and R. T. Schwartz, *N. Engl. J. Med.* **291**, 1304 (1974); V. Lennon, *Nature (London)* **258**, 11 (1975); D. B. Drachman, I. Kao, A. Pestronk, K. V. Toyka, *Ann. N.Y. Acad. Sci.* **274**, 226 (1976).
20. We thank Dr. M. Nirenberg, (National Institutes of Health, Bethesda, Md.) for the NG 108-15 clonal cells, Dr. A. M. Cohen for carrying out microcinematography, Dr. D. L. Rice for advice on autoradiography, and C. F. Barlow for assistance. This work was supported by research grants 5 PO1 NS10920, 5R01 HD04817, and an NIH academic career development award to I.K.

23 June 1976