RELATIONSHIP BETWEEN SUPERSENSITIVITY TO ACETYLCHOLINE AND PROTEIN SYNTHESIS IN DENERVATED RAT DIAPHRAGM MUSCLES

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Abstract—Correlation between denervation-induced ACh supersensitivity and protein synthesis was studied at cellular and tissue levels in tissues from rat diaphragms. Effects of unilateral denervation of diaphragm muscles on the incorporation of 14C-L-leucine into the total proteins were tested. After denervation, there were increases of 4.5 times within 2 days and double the increase was seen in 14 days in the rate of incorporation of 14C-L-leucine into the paralyzed hemidiaphragm. The administration of cycloheximide (1.0 mg/kg i.v.), in amounts that had experimentally hardly any effect on the incorporation into the normal muscle, suppressed their increases into the denervated muscles. From the rates of 3H-puromycin incorporation into the nascent proteins of microsomal fractions from both normal and denervated diaphragms, the reactivities of diaphragm microsomal fractions with puromycin were shown to be enhanced by denervation. In experiments on isolated muscle suspended in a bath containing modified Tyrode solution, the inhibitory effects of cycloheximide (CHI) on ACh submaximal contractions induced by supersensitivities after 5 days' denervation were depressed to a greater extent than were those after 2 weeks' denervation followed by injection of 1.0 mg/kg CHI into rat at one hour before removal of the diaphragm. These results indicate that denervation induces an increase in the rate of protein synthesis and that ACh supersensitivity is closely related to the enhanced rate of protein synthesis.

Acetylcholine (ACh)-receptors are confined to the myoneural junction and related areas, while after denervation, the region sensitive to ACh increases from the end-plate region until the entire surface on the muscle fiber becomes sensitive (1, 2, 3). One mechanism for the ACh supersensitivity may be the activation of normally latent receptors. Another possibility is that denervation may increase the rate of synthesis and/or reduce the rate of degradation for the ACh receptors possibly resulting in a more widespread localization on the muscle membrane.

Thus, changes in the protein composition of muscle of the rat in denervation (4), the effects of denervation on protein synthesis and the transport of amino acids in muscle (5), and increased incorporation of G-3H-leucine into a possible 'receptor' proteolipid in denervated muscle (6) were reported.

Although considerable attention has been given to protein as an ACh receptor, the relationships between ACh supersensitivity and increase of protein synthesis in cases of denervation have not been clarified.

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The present study concerns observations on both the increase of incorporation of $^{14}$C-L-leucine into total proteins induced by denervation and its suppression by blockers of protein synthesis. Details of the data in a previously published paper are also included (7).

**MATERIALS AND METHODS**

In a determined number of days before removal of the male rat diaphragm (200-400 g, Wistar), left unilateral phrenicotony was carried out by removing the phrenic nerve about 1-3 cm at the plexus cervicalis with the rats under ether anaesthesia. The procedures of incorporation of $^{14}$C-L-leucine were the same as those described in our previous paper (7).

The proteins were measured according to the method of Lowry et al. (8). The formation of peptidyl-$^3$H-puromycin was measured with microsomes (3 weeks' denervation) in incomplete mixtures (0.5 ml) that contained 40 mM Tris-HCl (pH 7.8), 80 mM KCl, 4 mM MgCl$_2$, $^3$H-puromycin (0.2-0.3 pM, 148,000-445 c.p.m. per pmole) and the microsomal fraction (105,000×g, 1 hr, 4 volumes of Medium M) (9) suspended in Medium A (10), and was also measured in a complete mixture (0.5 ml) that contained 0.55 mM GTP, 1 mM ATP, 10 mM sodium phosphoenolpyruvate, 0.02 mg pyruvate kinase, 2 mM mercaptoethanol, a mixture of 19 amino acids (each 0.3 mM), $^3$H-puromycin, the microsomal fraction and 0.05 ml liver supernate (105,000×g, 1 hr, 4 volumes of Medium A) in addition to the same composition as above described. Both mixtures were used to determine whether or not increase in the formation of peptidyl-$^3$H-puromycin might be dependent upon certain components in a complete mixture. After incubation at 37°C for 1, 2, 4 and 20 min, 5% TCA was added to the medium. After continuous washes with TCA, acetone, ethanol and ether cooled by ice, the samples were counted in a liquid scintillation spectrometer.

**Autoradiograph:** The method originated by Waser (11) was used. $^{14}$C-Leucine were given i.v. to mice with left lateral denervated diaphragms (11 days). After 4 min., the mice were decapitated and bled. The diaphragms were immediately removed, stretched on rings, and radioactively contaminated blood was removed. The thin preparations were dry within half a day and were then cut off the rings and brought into contact with X-ray films. After an exposure time of 49 days, the films were developed.

**Assay:** The denervated hemidiaphragms (0.5×3 cm) were suspended in an organ bath of 10 ml modified Tyrode solution at 36°C (12) oxygenated continuously by bubbling air. Isotonic contractures were recorded on a smoked kymograph drum with a lever (0.5 g weight and 25:1 ratio). Freshly prepared solutions of ACh were added to the bath at a constant volume (0.1 ml), and tissues were allowed to react for 30-60 sec. All contractions were expressed as percentages of the contracture produced at the end of each experiment by 0.1 M K$_2$SO$_4$ solution.

Rectus abdominis muscles of frog (Rana nigromaculata) were suspended in 5 ml of Ringer solution at a room temperature of 22-23°C. The contraction was expressed as percentages of that for ACh 1.64 mg/ml. Other experimental conditions were much the same as those in the case of diaphragm muscles.

**Drugs used:** Acetylcholine chloride (ACh) (Daiichi), actinomycin D (Act D) (Merck
and Dohme), cycloheximide (CHI) (Upjohn), uniformly labeled O-
and methyl-3H-puromycin (New England Nuclear) were used.

RESULTS

Effects of denervation on the incorporation of 14C-L-leucine into total proteins of rat
diaphragms: The rate of incorporation of 14C-L-leucine into total proteins of left lateral
denervated diaphragm was compared with that of right lateral innervated diaphragm as
shown in Fig. 1. The incorporation of 14C-L-leucine into diaphragms denervated for 2 days
showed the maximum increase (about 4 times) by comparison with its control and those for
2-4 weeks showed about 2-fold increases. There was a slight increase in the incorporation
of 14C-L-leucine into the right lateral intact diaphragm.

Effects of denervation on the incorporation of 3H-puromycin incorporation into micro-
somal fraction of diaphragm: The rate of incorporation of 3H-puromycin into subcellular
microsomal preparations of left lateral denervated diaphragms was compared with that of
right lateral innervated controls as shown in Fig. 2. As to the counts of 3H-puromycin
against mg protein and incubation minutes respectively, the incorporation into microsomal
fraction of denervated diaphragms (after 3 weeks) in both an incomplete and a complete
mixture was about twice that of innervated controls. The rate of incorporation of 3H-
puromycin into the subcellular microsomal fraction in the complete mixture was about
1.5-2 fold greater than that in the incomplete mixture.

Effects of cycloheximide on the rate of 14C-L-leucine incorporation into total proteins of
rat denervated diaphragms, and on denervation supersensitivity of diaphragm to acetylcholine:
In the diaphragms of rat successively administered CHI i.v., the rate of 14C-L-leucine in-
corporation into the denervated muscle decreased considerably in comparison to that of

![Fig. 1. Incorporation from 14C-L-leucine into total proteins of isolated denervated
rat diaphragm in 90 min. 14C-L-leucine was added to the incubation medium in
a diluted state to give a radioactivity of about 0.7 pCi/ml. Radioactivities (counts/
min incorporated/mg protein) of left lateral paralysed hemidiaphragms (○) and
right lateral innervated controls (●) against time (days) elapsed after phreni-
cectomy were plotted.](image)
Fits. 2. Rate of 3H-puromycin incorporation into the nascent proteins of microsomes from isolated denervated rat diaphragm. The microsomes were incubated with 3H-puromycin; in a complete mixture (0.5 ml) that contained 55 mM Tris-HCl (pH 7.8), 80 mM KCl, 4 mM MgCl₂, 0.55 mM GTP, 1 mM ATP, 10 mM sodium phosphoenolpyruvate, 0.02 mg pyruvate kinase, 2 mM mercaptoethanol, a mixture of 19 amino acids (each 0.3 mM), 3H-puromycin (0.3 μM, 445 c.p.m./μmol), the microsomal fraction (105,000 × g, 1 hr, Medium M) suspended in Medium A and 0.05 ml liver supernatant (105,000 × g, 1 hr, Medium A); in an incomplete mixture (0.5 ml) that contained 40 mM Tris-HCl (pH 7.8), 80 mM KCl, 4 mM MgCl₂, the same concentration of 3H-puromycin and the microsomes as in a complete mixture. Left figure shows the plots of total c.p.m. of 3H-puromycin incorporation in 20 min against mg protein. Right figure shows c.p.m. of 3H-puromycin incorporation per mg protein against time. (●) and (▲); left lateral denervated diaphragms. (●) and (▲); right lateral innervated controls. Circles; incomplete mixture. Triangles; complete mixture.

FIG. 3. Effect of cycloheximide administered once daily ×5 after denervation on the incorporation from 14C-L-leucine into total proteins of denervated diaphragms. Shaded columns; left lateral denervated diaphragms. Blank columns; right lateral innervated ones. Each column is the mean ± S.E. from 2–3 experiments paired with the experiments in Fig. 4.

Fig. 4. Log concentration-response curves of acetylcholine for denervated diaphragms of rats given cycloheximide once daily ×5 after phrenicectomy. Unilateral denervated diaphragms were isolated 5 days after phrenicectomy. Cycloheximide was given i.v. 20 or 10 μg/day/rat after phrenicectomy, alternatively, (●); treated with cycloheximide. (●); controls. Each point is the mean ± S.E. from 5 experiments.
control muscles as shown in Fig. 3.

The denervation supersensitivity of diaphragm to ACh was considerably depressed by CHI given i.v., once daily ×5 after denervation as shown in Fig. 4 and also once daily ×12 after denervation (data was not shown).

As a control experiment, the pharmacological effects of CHI and Act D were determined. The responses of denervated diaphragms (after 2 weeks) to ACh were not affected by the presence of CHI or Act D in the bath solution as shown in Fig. 5.

Effects of cycloheximide or actinomycin D on the rate of ¹⁴C-L-leucine incorporation into total proteins in intact and denervated diaphragms of rats: In the intact diaphragm from rats given CHI, the rate of ¹⁴C-L-leucine incorporation into the left lateral diaphragms was almost the same as that of the right lateral one (Fig. 6). The figure shows that the concentration of CHI used in the above experiments had almost no influence on the rate of ¹⁴C-L-leucine incorporation into the intact diaphragms.

In the diaphragms of rats given CHI or Act D, the rates of ¹⁴C-L-leucine incorporation into the denervated muscle were decreased considerably in contrast to that of control muscles, though there were no differences in those of innervated diaphragms from animals given or not given CHI or Act D, as shown in Fig. 7. The figure shows that CHI and Act D depressed the increased rate of incorporation into the denervated muscles to the innervated levels.

Effects of cycloheximide or actinomycin D on denervation supersensitivity of diaphragm to acetylcholine: The denervation supersensitivity of diaphragm (2 weeks after denervation) to ACh was not depressed by CHI given i.v. one hour before isolation of muscles, and was rather potentiated by Act D given 8 hr before isolation of muscles, as shown in Fig. 8. The
experimental condition in the way of administration of these blockers of protein synthesis into rats was the same as that in Fig. 7, and pharmacological supersensitivity to ACh was not

Fig. 7. Effect of cycloheximide and actinomycin D on the increase in incorporation from \(^{14}\text{C}-\text{L-leucine}\) into total proteins of isolated denervated diaphragms. Left lateral phrenicecctomy was carried out about 2 weeks before excision of the diaphragms. The intravenously given dose of cycloheximide and actinomycin D was the same as in Fig. 8. Shaded columns; left lateral denervated diaphragms. Open columns; right lateral innervated controls. Each column is the mean ± S.E. from 2 experiments.

Fig. 8. Log concentration-response curves of acetylcholine for denervated diaphragms of rat administered cycloheximide or actinomycin D once at 1 hr or 8 hr before excision of the diaphragm. Unilateral denervated diaphragms were isolated about 2 weeks after phrenicectomy. Cycloheximide (250 \(\mu\)g/rat i.v.) and actinomycin D (700 \(\mu\)g/rat i.v.) was given to rats 8 hr before excision of the diaphragm. (\(\triangle\)); treated with cycloheximide. (\(\bigcirc\)); treated with actinomycin D. (\(\bullet\)); controls. Each point is the mean ± S.E. from 4-7 experiments.

Fig. 9. Cumulative log concentration-response curves of acetylcholine for rectus abdominis muscles of frog in the presence (\(\bigtriangleup\)) and absence (\(\bullet\)) of actinomycin D (30 \(\mu\)g/ml) administered 1 hr before the administration of acetylcholine. Each point is the mean ± S.E. from 2 experiments.

Fig. 10. Autoradiograph of diaphragm 4 min after an intravenous administration of \(^{14}\text{C}-\text{L-leucine}\) (19.2 nM, 0.17 \(\mu\)Ci/g) to mice which had undergone left lateral phrenicectomy 11 days previously. Note the dark shade at a central zone, the region of end-plates, in the left lateral denervated side.
depressed in contrast to the incorporation of \(^{14}\)C-L-leucine into diaphragm suppressed by CHI or Act D, as shown in Fig. 7. Though the potentiation induced by Act D was not elucidated, it was clear that Act D had no antiChE action on rectus abdominis muscles of frogs as shown in Fig. 9.

Distribution of \(^{14}\)C-L-leucine incorporated into diaphragms and detected by autoradiographic techniques: The autoradiograph in Fig. 10 shows that the blackening of the left lateral denervated side was much more extensive than that of the right lateral innervated control.

The dark shadow produced by the incorporation of \(^{14}\)C-L-leucine is particularly concentrated in the endplate region. The shadow appears in the central zone of the left lateral denervated side denser than the other part at 48 hr, 7 days, 11 days (Fig. 10) and 14 days after denervation. These phenomena indicate that at some step in the process of denervation, the rate of protein synthesis of the end-plate region and its surroundings is greater as compared to that which occurs in other areas.

**DISCUSSION**

Elmqvist and Thesleff (12) reported that within 40 hr after denervation, contractures of rat diaphragms were not produced by ACh, and subsequently, however, graded contractures reached their maximum about 7 days after denervation. Manchester et al. (13) reported that RNA content of diaphragm increased considerably within 3 days after denervation and that incorporation of \(^{14}\)C-adenine and \(^{3}\)H-thymidine into DNA reached its maximum by about the third day. The mode of the increase of the rate of \(^{14}\)C-L-leucine into denervated hemidiaphragms as shown in Fig. 1 seems to be an initial phase with maximum increase at 2 days after denervation and a later plateau phase. The former phase suggests an increased rate of protein synthesis due to increase of RNA or DNA synthesis as reported by Manchester et al. (13) and the latter phase is possibly due to increase of receptor synthesis, because the denervation supersensitivity reached a maximum about two weeks after denervation.

In the above experiment, the increase of incorporation of \(^{14}\)C-L-leucine into total protein was not due to diminution of content of total protein of the diaphragm after denervation, because the enhanced reactivity of the diaphragm microsomal fraction with application of \(^{3}\)H-puromycin induced by phrenicotomy can be explained by an increase of nascent peptidyl-\(^{3}\)H-puromycin. Puromycin interacts with the binding sites of nascent peptide on ribosomes and is liberated from polyribosomes in the form of peptidyl puromycin. The increase of the rate of incorporation of \(^{14}\)C-L-leucine, therefore, was not due to \(^{14}\)C-leucyl-sRNA but rather to nascent polypeptides.

The supersensitivity of denervated diaphragms to ACh was not depressed by blockers of protein synthesis administered once at several hours before isolation of the muscles, but was depressed by the blockers administered once daily after denervation, suggesting that the bio-synthesis of ACh receptor protein may be completed in 5 days to 2 weeks by denervation and that the supersensitivity of denervated diaphragm muscles to ACh was not affected by the blocker of protein synthesis administered at several hours before isolation
of the muscles. On the other hand, the successive administration of the blocker to rats suppressed the biosynthesis of ACh receptor from the initial stage. These explanations are also supported by our experimental results that the heights of K+ contractures of denervated diaphragm muscles were not influenced by the blocker administered successively. ACh receptor proteins were apparently affected by the blocker, but not so the other muscle proteins which contributed to the contracture induced by K+ ion which served as the index of the maximum response of muscle contraction.

Fambrough (14) reported that neither Act D (1 \( \mu \)g/ml), CHI (1–100 \( \mu \)g/ml) and puromycin (10 \( \mu \)g/ml) had any effect on end-plate ACh sensitivity or on established extrajunctional ACh sensitivity by organ culture techniques. Grampp et al. (15) reported that treatment with Act D (0.5–1.0 mg/kg i.p.) on the day of denervation or the day following denervation prevented the fall in resting membrane potential, and the development of tetrodotoxin resistant action potentials and of extrajunctional cholinergic receptors in a majority of the fibers, whereas the treatment with Act D on the third day after denervation or thereafter had no effect on the time course of the development or on the intensity of denervation symptoms. These findings indicate that the administration design of protein synthesis inhibitor is most important, and we took special care that our experiments would be carried out carefully. According to the another paper by Grampp et al. (16), some denervation induced changes in muscle fiber membrane depend on the synthesis of new proteins. Our results are in good agreement with these findings.

The results of autoradiographic experiments showed that increase in the rate of the synthesis of new proteins is localized mainly in the end-plate region and its surroundings. As the increase in ACh sensitivity in denervated rat diaphragm muscle is the result of appearance of new ACh receptors in the extrasynaptic membrane (3, 14, 17), the proteins in these areas observed by the autoradiographic technique may be mainly composed of the extrajunctional receptor for ACh.

It is concluded that some denervation-induced changes in the muscle membrane, containing ACh receptor, depend on the synthesis of nascent protein. In this sense, the ACh receptors as well as control factors depending on the denervation may be considered to be protein-like.

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