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In Vitro Studies of Skeletal Muscle Membranes*

Adenylate Cyclase of Fast and Slow Twitch Muscle and the Effects of Denervation

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Summary. Sarcolemmal membranes were prepared from slow-twitch (red) and fast-twitch (white) skeletal muscle of the rat. A sensitive adenylate cyclase assay was used and basal, fluoride- and catecholamine-stimulated activities measured. The greater *in vivo* sensitivity of red muscle to the effects of catecholamines correlates, in the present study, with approximately a twofold stimulation of its sarcolemmal adenylate cyclase with isoproterenol (10 μM). The white muscle enzyme, on the other hand, is only minimally stimulated (20%) at the same concentration of β-adrenergic agonist. Fast-twitch muscle is known to be physiologically insensitive to catecholamine *in vivo*.

A course of sciatic nerve denervation was followed to further distinguish these two metabolic types of skeletal muscle and their respective adenylate cyclases. The slow-twitch muscle enzyme activities were completely and permanently lost on denervation. The white muscle enzyme, however, recovered almost completely after an initial reduction in specific activity the first week. Interestingly, the NaF-stimulated activity lagged behind both the basal and hormone-stimulated activities of the white muscle enzyme, in returning to control levels. The activities of cyclic nucleotide phosphodiesterase were evaluated in homogenates of the two muscle types in innervated rats and following denervation, in order to further define the neural influence on skeletal muscle cyclic nucleotide metabolism.

The results suggest that the motor nerve may regulate some of the metabolic properties of slow-twitch muscle (which may involve cyclic AMP) by controlling the responsiveness of its sarcolemmal-bound adenylate cyclase system.

The concept of "trophic" functions of neurons on target cells originated more than 50 years ago and continues to generate both interest and controversy (Guth, 1968; 1974). A recent symposium was entirely dedicated to it (Drachman, 1974). As a model of trophic action, the influence of nerve on skeletal muscle has been the subject of many recent investigations (Redfern & Thesleff, 1971; Albuquerque, Warnick,

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Tasse & Sansone, 1972; Lomo & Rosenthal, 1972; Jones & Vrbova, 1974). The neural regulation of fast-twitch and slow-twitch physiological and metabolic properties has been well-documented, but much less is known about the molecular mechanisms controlling influences on morphology (Tower, 1935), acetylcholine sensitivity (Axelsson & Thesleff, 1959; Lomo & Rosenthal, 1972), resting membrane potential (Albuquerque *et al.*, 1971), cholinesterase activity (Guth, 1968) and myosin light chains (Guth, 1968; Close, 1972). Many of the differences between fast-twitch and slow-twitch muscle have been used to support the neurotrophic role of the motor nerve. Known differences in physiologic and metabolic responses to catecholamines (Bowman & Nott, 1969) of "fast" and "slow" muscles and the acknowledged role of the motor nerve in determining a number of muscle properties of these two major types of skeletal muscle suggest the possibility of some intermediate(s), originating at the plasma membrane, integrating these various effects.

It has been demonstrated that in addition to acetylcholine (ACh) and ATP (Silinsky & Hubbard, 1973) a protein (Musick & Hubbard, 1972) is released at neuromuscular junctions. If this protein is a "trophic factor", perhaps similar to that found by Oh (1975) to stimulate differentiation of chick muscle in culture, it should interact with some "receptor(s)" at the surface of the muscle cell, with its effect mediated via a change in intracellular levels of some molecule(s). Lentz (1972) and recently Carlsen (1975) propose that this may occur via a change in adenosine 3′-5′ cyclic monophosphate (cAMP) levels. McMahon (1974) has reviewed a large body of data and has hypothesized a variety of roles for cyclic AMP as inter- and intracellular messenger, along with Ca^{++} and catecholamine neurotransmitters.

The effects of catecholamines on skeletal muscle acting at β-receptor sites should be mediated, as in other tissues, via an increase in cAMP production. This occurs, presumably, by stimulation of the adenylate cyclase system of muscle (Sutherland & Robison, 1966: Lefkowitz, 1973). At precisely what intracellular locus or sites cyclic AMP exerts its effects are not completely known at this point. Therefore, we have chosen to evaluate the membrane-bound adenylate cyclase system in both muscle types to explore the role of the nerve on a definable muscle membrane characteristic. In the present report, we document qualitative as well as quantitative differences in purified sarcolemmal (SL) membrane adenylate cyclase of normal and denervated soleus (slow-twitch) and extensor digitorum longus (EDL: fast-twitch) muscles in the rat. The effects of denervation on the macromolecular components of Na^+ and K^+

transport in skeletal muscle (NaK-ATPase, [^{32}P]-incorporation, [^{3}H]-ouabain binding), as well as the SL protein patterns on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), are presented in an accompanying paper (Festoff, Oliver & Reddy, 1977). Parallel studies on alterations in the intracellular levels of cAMP and cGMP will be reported elsewhere. A preliminary report of the present studies has appeared in an abstract form (Reddy, Oliver, Engel & Festoff, 1976*b*).

Materials and Methods

Sarcolemmal membranes were prepared essentially as reported previously (Festoff & Engel, 1974). The soleus, EDL and gastrocnemius muscles of male Sprague-Dawley rats were isolated, dissected free of connective tissue, nerves and blood vessels and homogenized as described (Reddy, Engel & Festoff, 1976*a*). Denervation was performed by resection of 1 cm of sciatic nerve 2 cm proximal to its innervation of muscles. The opposite unoperated leg served as control for all time points.

Adenylate cyclase assay was performed using [α-^{32}P]-ATP according to the method of Salomon, Londos and Rodbell (1974). In brief, assay tubes, in a final volume of 0.1 ml, contained the following (μmoles): [α-^{32}P]-ATP, 0.15 (1×10^6 cpm); $MgCl_2$, 0.5; KCl, 1.0; theophylline, 1.0; creatine phosphate, 1.8; 28 μg CPK; and 50–80 μg of SL membrane protein. Tubes were incubated at 37 °C for 15 min in a shaking water bath and the assay terminated by addition of stopping mix [2% SDS, 1 mM ATP, 1 mM ^{3}H-cyclic AMP (8000 cpm)]. Separation of newly formed ^{32}P-cyclic AMP was by sequential elution on Dowex-50 and alumina gel columns (Salomon *et al.*, 1974). To the final 5 ml elution volume (2 ml H_2O, 3 ml 0.1 M imidazole), 15 ml Aquasol (NEN) were added and vials were counted in a Beckman LS-355 scintillation counter optimized for dual-label ^{3}H and ^{32}P counting. Recoveries for [^{3}H]-cAMP ranged from 60 to 85%.

Assay of cyclic nucleotide phosphodiesterase (PDE) was performed according to the method of Thompson, Brooker and Appleman (1974). Assay tubes, in a final volume of 0.2 ml, contained 5 mM $MgCl_2$, 1 μM or 200 μM ^{3}H-cyclic AMP (2.5×10^5 cpm at 40% efficiency), 40 mM Tris-Cl, pH 8.0, and 50 μl of a 10% (w/v) homogenate of fast-twitch or slow-twitch muscle from normal or denervated hind legs. Sample and control (boiled or no homogenate) tubes were incubated for 30 min at 30 °C in a shaking water bath. Counts obtained in control tubes were always less than 2% of the added radioactivity and were subtracted from the experimental values. Enzyme activity is expressed as nmoles cyclic AMP hydrolyzed per min per mg of noncollagenous protein (NCP).

Protein was estimated by the method of Lowry, Rosebrough, Farr and Randall (1951) using bovine serum albumin as standard. NCP was estimated in homogenates as previously described (Lilienthal, Zierler, Folk, Buka & Riley, 1950). [α-^{32}P]-ATP (10–30 Ci/mmole) and [^{3}H]-cyclic AMP (36 Ci/mmole) were obtained from New England Nuclear (Boston, Mass.). Unlabeled nucleotides were obtained from Sigma (St. Louis, Mo.) or Calbiochem (La Jolla, Calif.). All other chemicals were of the highest commercial purity available.

Results

Effect of Denervation on Bulk of "Fast" and "Slow" Muscle

Confirming a number of previous studies (Gutman & Zelena, 1962), denervation of hind-limb musculature (proximal denervation – severance

Table 1. Effect of proximal denervation on wet weight of "fast," "slow," and "mixed" muscles of the rat

Weeks of denervation	Soleus	% Loss	EDL	% Loss	Gastroc	% Loss
1	2.09 (3.42)	38	2.96 (4.18)	28	30.60 (45.08)	31
2	1.56 (3.70)	53	2.80 (4.40)	50	22.40 (51.30)	54
5	1.60 (4.40)	64	2.30 (5.80)	60	14.90 (54.70)	73

Values are in grams wet weight. Normal (control leg) muscle weights are in parentheses.

of sciatic nerve 1 cm proximal to the popliteal fossa) resulted in significant loss of muscle bulk, as shown in Table 1. The largest single percentage decrement occurred in the "slow" soleus ("red" or "tonic") at seven days post-denervation, although "fast" ("white" or "phasic") and mixed-type muscles all suffered considerable loss of mass over the five-week period compared with controls.

Our previous studies (Festoff & Engel, 1974; Reddy *et al.*, 1976*a*) with isolated sarcolemmal (SL) membranes were directed at characterizing extrajunctional, or more physiologically conductile, as well as junctional membrane components. In the present studies, we have evaluated the specific activities of these components in denervated as well as normal membranes hoping to detect any characteristic suggesting neurotrophic control. It should be kept in mind that a post-denervation change, especially of a quantitative nature in a muscle membrane characteristic does not necessarily indicate that it is neurotrophically regulated.

Denervation Effects on "Fast-Twitch" Muscle Adenylate Cyclase

The basal, fluoride- and catecholamine-stimulated values of normal fast muscle (EDL) adenylate cyclase are less than the respective values for the "slow" muscle (soleus) enzyme (Table 2). Only minimal activation (20%) occurs with isoproterenol. A dramatic reduction of all these parameters takes place by the first week post-denervation (Fig. 1). The values, expressed as percentage maximum (control), show a 60 to 70% reduction in specific activity after seven days of nerve section. The time course of the effects of loss of neural influence on EDL sarcolemmal adenylate cyclase over the next month is even more interesting. By 14 days, there is a tendency for the basal and isoproterenol-stimulated activities to return to normal and appear to remain at near normal levels at five weeks post-denervation. The catalytic reactivity, as estimated by the re-

Table 2. Adenylate cyclase activities of "fast" and "slow" sarcolemmal membranes

	Soleus	EDL
Basal	16.8± 4.5	10.3± 1.1
Isoproterenol	31.8± 5.8	12.1± 2.3
NaF	143.0±34.3	112.0± 32.2

Assay tubes contained (mM): ATP, 0.5; $MgCl^{++}$, 5; KCl, 10; theophylline, 10; Tris HCl (pH 7.5), 50; and 30–40 μg SL protein. Assay of newly formed ^{32}P-cAMP as described by Salomon *et al.* (1974) in Materials and Methods. Specific activities are p moles × min^{-1} × mg $protein^{-1}$. Values are means ± SE of triplicates of four different preparations.

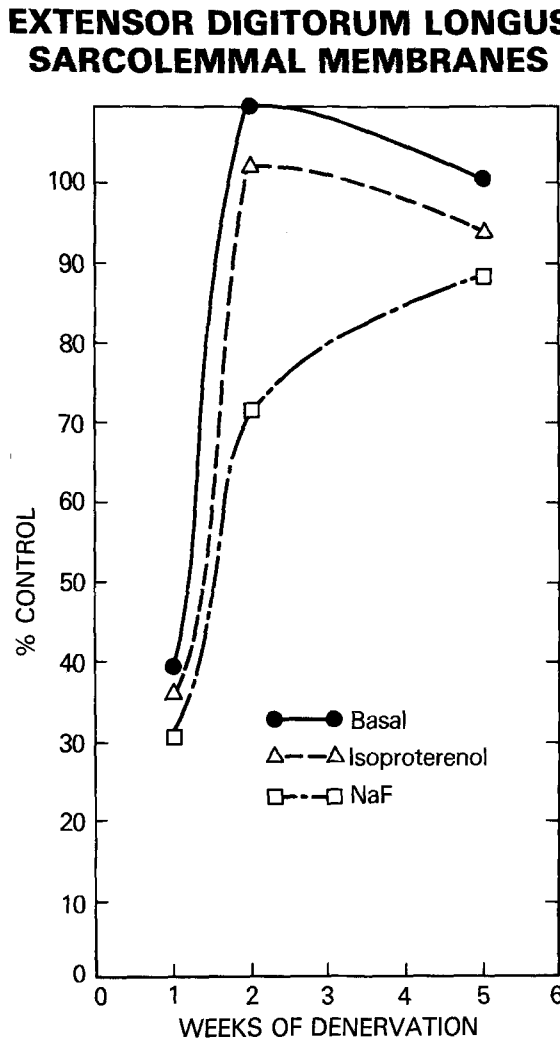

Fig. 1. Effect of denervation on "fast" muscle sarcolemmal adenylate cyclase. Assays as described in Materials and Methods. Points are means of three different experiments performed in triplicate; values for triplicates were within 5%

sponse to NaF, appears to lag behind. The return to control values occurs despite the previously mentioned 60% reduction in muscle weight at that time. It is well appreciated that "chronic" denervation primarily results in a loss of muscle myofibrillar protein structures, not membrane protein, and an increase in collagenous (connective tissue) protein (Zak, 1962). In the membrane fraction, one would not expect to recover collagenous protein material so that a maintenance of near-normal specific activity implies significant restoration, approaching the innervated state. Just what influence the nerve has on this muscle membrane component, identifiable within one week after disrupting the physical and electrochemical interaction between the two, remains to be elucidated. Fluoride-activated adenylate cyclase activity has been reported in aneurally grown chick muscle in culture (Zalin & Montague, 1974), and we have recently found that similar cultures also possess demonstrable catecholamine-stimulated activity *(unpublished)*. Therefore, in the absence of any direct neural influence, muscle adenylate cyclase will respond to hormones. However, once this neural influence is established and then removed, considerable alteration in activity results. This is even more obvious in the case of "slow" muscle cyclase activity.

Denervation Effects on "Slow" Muscle Adenylate Cyclase

As discussed above, higher basal levels as well as fluoride and hormone stimulation are found in the cyclase of normal "slow" SL compared with EDL membranes (Table 2). There are differences, as well, with the degree of activation with NaF and isoproterenol. Adenylate cyclase activity of soleus SL was stimulated almust twofold by isoproterenol whereas only a 20% increment was observed with that of EDL. The percentage of stimulation by fluoride, on the other hand, was slightly greater in the EDL cyclase compared with that of the soleus enzyme. What is interesting, and possibly more significant, is the time-dependent pattern of the denervation effect on soleus SL cyclase. As with EDL, marked reduction in adenylate cyclase activities, in all parameters, occured within seven days of denervation. Only 25 to 38% of these activities are present after one week. However, contrary to "fast" muscle, where restorative patterns appear after the first week (Fig. 1), these "slow" muscle membranes do not regain, and in fact continue to lose, specific activity (Fig. 2). By the fifth week post-denervation, 85 to 95% reduction in all activities is found. These results are quite striking and suggest that, at least in terms of adenylate cyclase activity, considerable

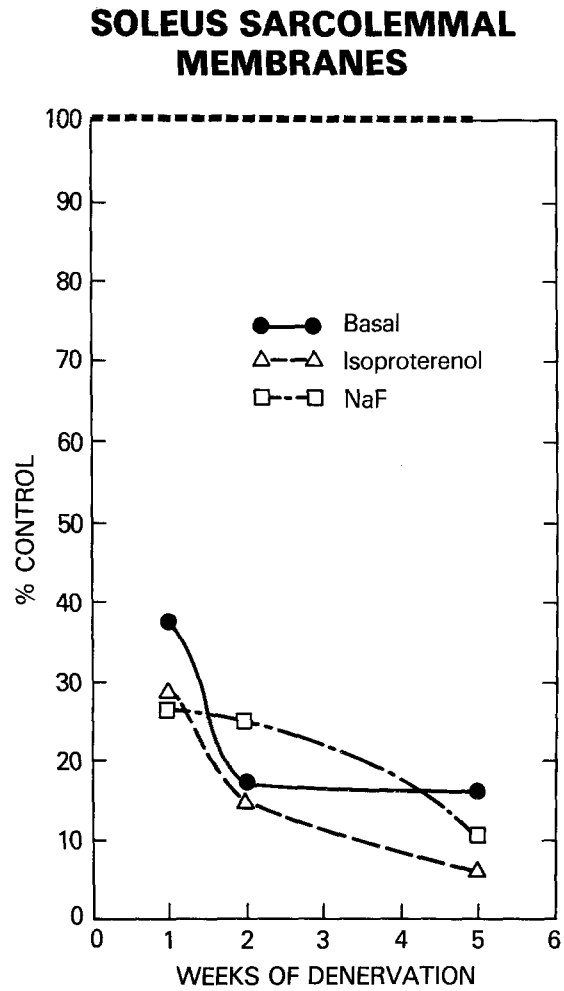

Fig. 2. Effect of denervation on "slow" muscle sarcolemmal adenylate cyclase. Assays performed as described in Materials and Methods. The points represent the means of three different experiments performed in triplicate; values for triplicates usually were within 5%

"trophic" influence is exerted on "slow" muscle by its innervating nerve. Whether these results are in conflict with the notion that certain properties of fast-twitch muscle, rather than tonic or slow-twitch skeletal muscle, appear to be the more dependent on neural influence (Albuquerque, Schuh & Kaufman, 1971; Close, 1972) must await further clarification.

Effects of Denervation on Cyclic Nucleotide Phosphodiesterase Activities

In most mammalian cells, there are at least two distinct cyclic nucleotide PDE (Thompson & Appleman, 1971). Greater or lesser affinities

Table 3. PDE activity of red and white muscle homogenates after denervation

Muscle	Time after denervation (days)	PDE[a]	
		Low K_m	High K_m
Soleus	7		
Control		8.84±1.40	152±26
Denervated		10.88±0.86	170±15
	14	(NS)	(NS)
Control		8.50±0.60	149±16
Denervated		13.14±1.40	190±12
		($p<0.005$)	($p<0.01$)
EDL	7		
Control		10.20±1.90	165±22
Denervated		12.05±0.28	174±11
		(NS)	(NS)
	14		
Control		9.50±0.64	158±19
Denervated		15.20±0.82	224±18
		($p<0.001$)	($p<0.01$)

[a] Specific activity = p moles cyclic AMP hydrolyzed per min per mg NCP. Values are means of triplicate determinations of three different preparations ×SEM. The low and high K_m PDE activities were determined at substrate concentrations of 1 and 200 μM, respectively. NS = not significant by Student's *t*-test.

are demonstrated for cyclic AMP and GMP, respectively. In addition, both high K_m and low K_m PDE have been found, at least for cyclic AMP (Thompson & Appleman, 1971; Thompson *et al.*, 1974). The low K_m (high affinity) enzyme has been found to be associated with particulate or membrane fractions of broken cell preparations. Because of the marked change in adenylate cyclase activities following denervation and the dramatic difference in response of red versus white muscle SL membranes, we evaluated the PDE in homogenates of the respective muscle types at seven and 14 days following denervation. The results of these experiments are shown in Table 3 and are expressed per mg of NCP. NCP was used because of the known increase in collagenous protein occurring in muscle homogenates following denervation (Zak, 1962). Both the low and high K_m PDE activities increased slightly after one week but statistically significant increase was found after two weeks of denervation in both the muscles.

The cyclic nucleotide levels in the muscle fiber at any stage of innervation or denervation should reflect a steady state and balance of the adenylate cyclase and PDE activities. Therefore, these data and the cyclase results should provide a picture of the effects on cyclic nucleotide

metabolism in muscle. However, they do not immediately reveal the role of the motor nerve in this extremely important metabolic system of muscle.

Discussion

The effects of catecholamines acting on β-adrenergic receptors appear to be mediated via an increase in cAMP production, presumably by stimulating membrane-bound adenylate cyclase (Sutherland & Robison, 1966; Lefkowitz, 1973). The physiological effects of catecholamines on "fast" and "slow" skeletal muscles have been reviewed by Bowman and Nott (1969). Epinephrine and isoproterenol increase both the twitch tension and total duration of the twitch as well as cause a delay in the time to peak tension in white muscle. However, the doses responsible are in the pharmacologic rather than physiologic range. In contrast, the effects of catecholamines on red muscles are just the opposite. Isoproterenol and epinephrine, presumably acting again via β-receptors, decrease the twitch tension and shorten the duration of twitch (i.e., increase the rate of relaxation) of "slow" muscle. In this respect, red muscle resembles cardiac muscle in its responses (Lefkowitz, 1973). These effects are produced at doses that are in the physiologic range, suggesting that an inherent increase in sensitivity to catecholamines is found in "slow" skeletal muscle and that, if mediated via cAMP, the nucleotide has qualitatively different effects in "fast" and "slow" muscles (Bowman & Nott, 1969). These effects on twitch and twitch duration for both "fast" and "slow" muscles have been ascribed to alterations in the active state. Catecholamines prolong the active state in "fast" muscle while curtailing this property in slow muscle (Goffart & Ritchie, 1952; Bowman & Raper, 1962). Denervation-induced changes such as fibrillation potentials (Salafsky, Bell & Prewitt, 1968) and reduced rate of rise of the action potential (Redfern & Thesleff, 1971) have also implicated prolongation of active state in "fast" muscle.

We have attempted to carry out an analysis of cell-cell interaction *in vitro* using a "model" excitable membrane, the skeletal muscle sarcolemmal complex. These studies, though preliminary, suggest that significant and specific denervation-induced alteration occurs in selective functional muscle membrane components and that qualitative as well as quantitative differences are apparent in the responses of "fast" and "slow" muscles to this alteration.

In our present studies, we have demonstrated higher basal values in slow-twitch muscle SL membrane adenylate cyclase when compared

with the fast-twitch muscle enzyme. More important, the soleus membranes were stimulated almost twofold by isoproterenol, whereas EDL cyclase activation was only about 20% (Table 2). This finding correlates with the increased sensitivity of red muscle to catecholamine *in vivo*.

In the denervated state, fibrillation potentials develop quite early and, at least in the rat, unequally in "fast" and "slow" muscles relative to the level of denervation (Salafsky *et al.*, 1968). Soleus spontaneous fibrillation activity is more vigorous than "fast" muscle (Bowman & Raper, 1965) and responds to a lesser dose of catecholamine. This response is qualitatively the same in denervated "fast" and "slow" muscles, i.e., an increase in frequency of fibrillations and muscle tone, whereas it is different in normally innervated muscles. Following denervation, therefore, the effect of catecholamines on "slow" muscle is to decrease its rate of relaxation. This is the effect in normally innervated "fast" muscle and just opposite of its role in normal "slow" muscle, where an increased rate of relaxation is observed. The reduction in specific activity of adenylate cyclase and lessened response to isoproterenol at one week tends to decrease further over the course of chronic denervation in the soleus muscle membranes (Fig. 2), whereas an attempt to restoration occurs with EDL membrane adenylate cyclase (Fig. 1). The effects of cross-reinnervation on the action of catecholamines on SL adenylate cyclase and the binding of β-receptor blocking agents would be of interest here.

The *in vivo* effects at first appeared inconsistent with our current findings with SL membranes. It was considered possible that a change in the sedimentation characteristics of membranes occurred in chronic denervation with resultant alterations in partitioning. In this regard, recent experiments with crude homogenates show a marked increase in catecholamine-responsive adenylate cyclase of soleus muscle two days post-denervation while dropping to below control levels later *(unpublished)*. No such increase occurs with homogenates of EDL. This increase in cyclase in soleus homogenates at two days may correlate with the observed rise in intracellular cAMP 48 hours after denervation, as reported by Carlsen (1975). It is also of interest that an increase in muscle glycogen content has been observed in the rat at two days post-denervation followed by a sharp drop in these levels with the development of spontaneous fibrillations (Gutmann & Zelena, 1962).

We attempted to further pursue these different physiologic responses by measuring PDE activities of muscle homogenates. It appears that the first four days following denervation may be important since this

period shows qualitative differences in red and white muscle (not shown). Both low and to a lesser extent high K_m PDE drops in EDL. In soleus, it is more complex and suggests a biphasic response during the first four days after denervation (not shown). However, as shown in Table 3, PDE activities of both the muscle homogenates showed a significant increase two weeks after denervation. It is of interest to note that at the same period of denervation the adenylate cyclase activity of sarcolemmal membranes showed a differential response (Figs. 1 and 2). These results suggest differential effects of denervation on the PDE and adenylate cyclase activities in these two types of muscles.

It is possible that denervation in red muscle has altered the metabolic responsiveness to catecholamine so that the effects of the neurotransmitter are not mediated via the adenylate cyclase-cyclic AMP system. This may be the mechanism by which epinephrine affects normal fast-twitch muscle at high concentrations. If this is true then it is imperative to search for catecholamine-responsive and cyclic AMP-mediated metabolic processes in slow-twitch muscle which may be under neurotrophic control and which may also be defective in human neuromuscular diseases where hormone unresponsiveness of muscle membrane adenylate cyclase has been demonstrated (Mawatari, Takagi & Rowland, 1974; Susheela, Kaul, Sachdeva & Singh, 1974; Canal, Frattola & Smirne, 1975). One such candidate may be a hormone-sensitive lipase because of its potential role in highly oxidative red muscle. Janaki and Susheeela (1966) have demonstrated histochemically a deficiency of lipase in muscle of patients with Duchenne dystrophy. Evaluation of lipase in both metabolic types of muscle in the innervated state and following denervation is in progress. Current experiments utilizing the binding of high affinity, stereo-specific β-antagonists (Aurbach, Fedak, Woodard, Palmer, Hauser & Troxler, 1974) are expected to help resolve these apparent discrepancies.

In summary, using a relatively purified sarcolemmal membrane preparation, we have analyzed the effects of denervation on a definable membrane-oriented function *in vitro*. Since neurotrophic regulation has been proposed as being the basis for many of the differences between "fast" and "slow" muscles, we have sought to determine if membrane differences also exist. In the studies presented in this report, qualitative as well as quantitative differences were found in adenylate cyclase activities of soleus and EDL membranes. Those differences, primarily in catecholamine-stimulated cAMP production, may relate to the observed differences in responses to these drugs *in vivo*. Differences were also found in the response of "fast" and "slow" adenylate cyclase to loss of neural in-

fluence. The "fast" muscle cyclase, shown normally to have little capacity to respond to catecholamines, initially drops at one week then returns to "control" levels by one month. In contrast, the catecholamine-sensitive "slow" adenylate cyclase was markedly and persistently reduced to barely assayable levels after denervation.

In the final analysis, it remains to be proved whether any of these changes represent "neurotrophic" regulations. They do suggest, however, that skeletal muscle membranes provide a convenient model system for the *in vitro* analysis of normal and disordered intercellular communication in the nervous system.

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