Electrical Properties and Acetylcholine Sensitivity of Singly and Multiply Innervated Avian Muscle Fibers

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ABSTRACT Membrane constants and distribution of acetylcholine (ACh) receptors were determined for multiply innervated fibers of the anterior latissimus dorsi (ALD) and singly innervated fibers of the posterior latissimus dorsi (PLD) muscles of 3–6 month old chickens. The values of the various membrane constants were: length constant, 1.78 mm (mean) in ALD, 0.68 mm in PLD; time constant, 35 msec in ALD, 3.7 msec in PLD; transverse membrane resistance, 4388 Ω cm^2 in ALD, 561 Ω cm^2 in PLD; and membrane capacitance, 8.2 $\mu F/cm^2$ in ALD, 7.0 $\mu F/cm^2$ in PLD. Peaks of ACh sensitivity occurred at intervals of ca. 740 μ on ALD fibers with a low sensitivity remaining between peaks. Only one peak of ACh sensitivity was detected on PLD fibers. The maximum ACh sensitivity found was 5 ± 4 mv/ncoul for fibers of the ALD and 77 ± 60 mv/ncoul for fibers of the PLD. The distance over which this sensitivity fell to 0.1 was ca. 225 μ in the ALD and 140 μ in the PLD. The membranes of these two muscle fiber types differ widely regarding some electrical properties and the disposition of ACh-sensitive receptor sites.

INTRODUCTION

The anterior and posterior latissimus dorsi (ALD and PLD) muscles of the chicken provide a unique experimental preparation for studying the possible relationships between the type of motor innervation and the membrane characteristics of muscles. All fibers of the ALD possess multiple "*en grappe*" innervation points at ca. 1 mm intervals along their length (Hess, 1961, 1967; Ginsborg and Mackay, 1960; Mayr, 1966) and contract slowly to indirect stimulation (Ginsborg, 1960 *a*). Most fibers of the PLD, on the other hand, possess a single innervation point with an "*en plaque*" type of junction (Hess, 1961, 1967) and contract with a twitch to indirect stimulation (Ginsborg, 1960 *a*). The magnitude of the transverse membrane resistance (R_m) for fibers of the rat diaphragm (unpublished data) is very similar to that found

for the soleus and extensor digitorum longus of the rat (Kiyohara and Sato, 1967), and the tenuissimus of the cat (Boyd and Martin, 1959). These muscles all possess a focal *en plaque* type of innervation with one, or at least very few, innervation points per fiber. On the other hand, tonic fibers of the iliofibularis of the frog, which possess multiple innervation points of the *en grappe* type, have values of R_m about nine times higher than that of the focally innervated twitch fibers of this muscle (Adrian and Peachey, 1965).

Cross-innervation experiments have demonstrated that both structure and function of a muscle depend on its type of motor innervation (Buller et al., 1960; Guth et al., 1968). It is well-known that motoneurons influence the nature of muscle fiber membranes. The magnitude of membrane resistance, as well as the degree of dispersion of chemosensitive receptor sites on the membrane is dependent upon intact innervation (Nicholls, 1956; Diamond and Miledi, 1959; Miledi, 1963). It was the aim of the present study to determine membrane electrical constants and distribution and properties of acetylcholine receptors of muscle fibers with widely differing types of innervation.

METHODS

Preparation The ALD and PLD muscles were removed from 3–6 month old male white Leghorn chickens in the manner described by Ginsborg (1960 *a*). The muscles were kept moist during removal, pinned at their approximate in situ length in a Petri dish containing a plastic resin (Sylgard 182 encapsulating resin and curing agent, Electronic Materials Department, Dow Corning Corp., Midland, Mich.), and bathed in a solution of the following composition (mM): NaCl 150, KCl 5.0, $NaHCO_3$ 20, $MgCl_2$ 2.0, $CaCl_2$ 2.4, glucose 21.1. Experiments were conducted at room temperature (20–23°C). In some cases, 95% O_2–5% CO_2 was bubbled through the solution during removal of connective tissue and during the experiment.

In most cases, the deep surface of the muscle was used. Connective tissue was removed with the aid of a dissecting microscope. Damage to some of the superficial fibers was evident as judged by their low resting potentials. The preparation was viewed with the aid of transmitted illumination and a dissecting microscope (up to 50 ×) equipped with an ocular micrometer for measurement of interelectrode distance.

Membrane Constants Membrane constants were obtained from measurements of transmembrane potential changes and of current pulses applied through a microelectrode inserted at various distances from the intracellular recording electrode (Fatt and Katz, 1951; Boyd and Martin, 1959). Microelectrodes were filled with 3M KCl solution and had resistances of 15–40 MΩ. A 100 MΩ resistor was placed in series with the current-passing microelectrode. Current intensity was measured by recording the voltage drop across a 10 KΩ resistor in the current-passing circuit. Artifact induced in the recordings by interelectrode capacitance was reduced with a grounded shield placed between the microelectrodes and a grounded tubular shield around the current electrode. Recordings of transmembrane voltage change and current were displayed

on a dual beam oscilloscope (Tektronix 502) and filmed with a Grass camera (Model C-4, Grass Instruments, Quincy, Mass.).

The recording electrode was first inserted into a muscle fiber and the transmembrane potential was noted. The current electrode was then inserted into the same cell, as indicated by the appearance of an electrotonic potential, within 50 μ of the recording electrode. There was usually a drop in resting potential of a few millivolts following insertion of the current electrode. Following a measurement at this position, the current electrode was inserted at a more distant position, usually about 1 mm from the recording electrode. The current electrode was then inserted about midway between the first two insertions, and a third measurement was taken. Frequently, especially in fibers of the ALD, there was a large drop in resting potential of 10–15 mv following the third insertion of the current electrode. Measurements were discarded when such a drop in potential occurred and when a semilog plot of V/I *vs.* interelectrode distance was not a straight line (Fig. 2). This difficulty was less frequently encountered in fibers of the PLD, even though no appreciable difference in diameter of fibers from the two muscles was found.

ACh Sensitivity and Desensitization Sensitivity to acetylcholine (ACh) and desensitization of ACh receptors were studied by recording transmembrane potentials with an intracellular micropipette while applying ACh iontophoretically to the membrane surface with another micropipette filled with approximately 2 M AChCl solution (del Castillo and Katz, 1955; Miledi, 1960 *a*). The ACh electrode was advanced toward the muscle membrane until a slight change in transmembrane potential was observed as the ACh pipette touched the membrane.

The strength and duration of the current pulse through the ACh electrode were adjusted until a depolarization of a few millivolts was produced. The electrode was then moved to various membrane sites and the process repeated. The pulse duration was varied from 0.2 msec at sensitive spots to 440 msec at insensitive spots. If no detectable membrane response was obtained with these long current pulses (1×10^{-7} amp for 440 msec), the membrane was considered to be insensitive to ACh.

An indication of the degree of desensitization of a membrane site was obtained by repeated application of ACh to a site while recording the change in ACh depolarization. If the magnitude of the depolarization returned to its original level after a few minutes rest period during which no ACh was applied, it was considered that the change in depolarization resulted from desensitization and not from movement of the ACh electrode.

Histology Measurements of fiber radius were made from histological preparations of the ALD and PLD from one 4-month old white Leghorn male. A piece of each muscle was frozen in 2-methylbutane cooled at -125°C in liquid nitrogen. Transverse sections 10 μ thick were cut on a cryostat at -15° to -20°C, then fixed in 15% formal saline and stained with hematoxylin and eosin. A cross-sectional area of 200 fibers from each muscle was measured from photographic enlargements with a planimeter. These measurements were then converted to fiber radius, assuming the fibers to be circular.

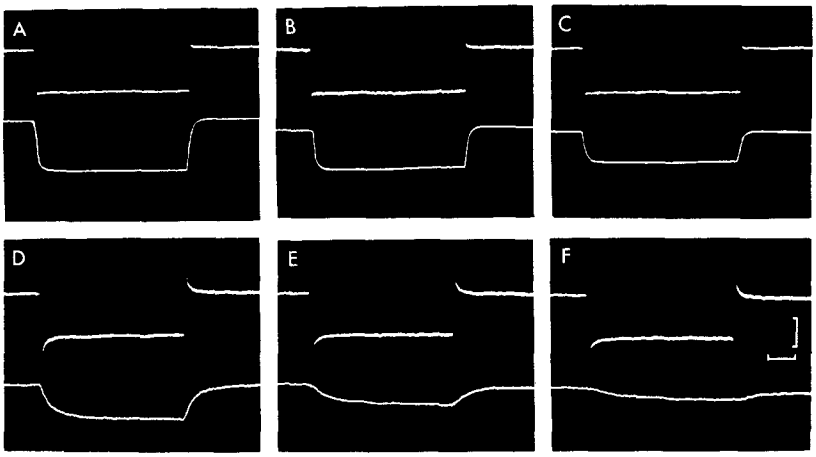

FIGURE 1. Hyperpolarizing electrotonic potentials produced by current pulses applied at three different distances. A, B, and C are from a fiber of the ALD; D, E, and F from a fiber of the PLD. The decrement in electrotonic potentials with distance was much less in fibers from the ALD than in fibers from the PLD. Upper trace in each record is current pulse. Interelectrode distances were: A, 50 μ; B, 460 μ; C, 960 μ; D, 50 μ; E, 440 μ; F, 1000 μ. Vertical calibration is 10 mv for A, B, and C, 5 mv for D, E, and F, and 2×10^{-8} amp. Horizontal calibration is 100 msec for A, B, and C and 5 msec for D, E, and F. Bath temperature 22°C.

RESULTS

Membrane Constants Electrotonic potentials recorded with the current electrode at three different distances from the recording electrode in fibers of the ALD and PLD showed marked differences in the degree of decrement with distance (Fig. 1). Electrotonic potentials from the ALD were larger for a given current pulse and had a much slower rise time (note the 20-fold time scale difference). A semilogarithmic plot of V/I *vs.* interelectrode distance (Fig. 2)

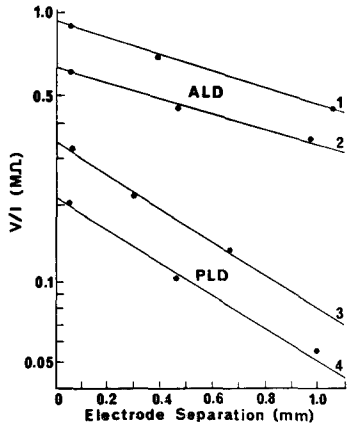

FIGURE 2. Semilogarithmic relation between V/I and electrode separation for fibers from the ALD and PLD. The plots for fibers 2 and 4 are from the tracings shown in Fig. 1. Resting potentials after first insertion of current microelectrode were 1, 50 mv; 2, 60 mv; 3, 55 mv; 4, 60 mv. Bath temperature 22°C.

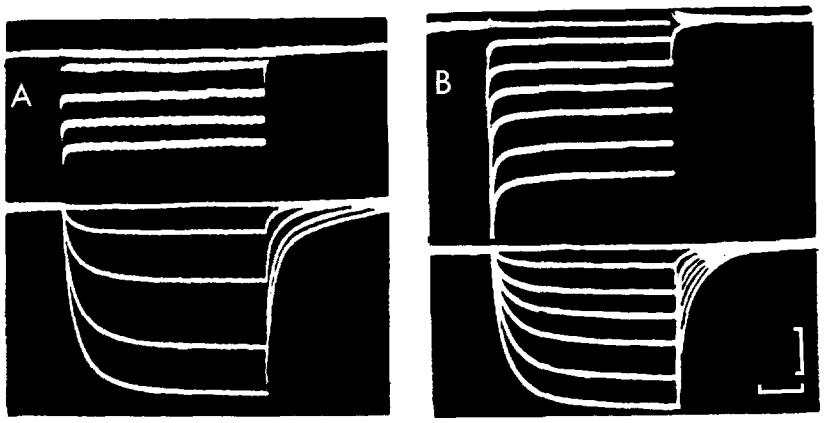

FIGURE 3. Current-voltage relationship for fibers from the ALD (A) and PLD (B). Top traces, current; bottom traces, membrane potential. Hyperpolarizing pulses, bath temperature 22°C, electrode separation less than 50 μ. Vertical scale: 10 mv for both A and B; 2×10^{-8} amp for A; 5×10^{-8} amp for B. Horizontal scale: 100 msec for A; 5 msec for B.

demonstrated a marked difference in length constant, λ, and in "input resistance," V/I at an electrode separation of zero millimeters, of fibers from these two muscles. This plot was derived by applying the cable theory equation $V = \frac{1}{2} I \sqrt{(r_m r_i)} \exp\left[-x/\sqrt{(r_m/r_i)}\right]$ (Hodgkin and Rushton, 1946) where V is the potential change produced by a steady current, I, through the membrane, x is the electrode separation, r_m is the transverse resistance of a unit length of membrane, and r_i is the internal longitudinal resistance per unit length of fiber. The term $\sqrt{(r_m/r_i)}$ is the space constant, λ, and is the distance from any point on the fiber over which the electrotonic potential falls to $1/e$ of its value at that point. The term $\frac{1}{2}\sqrt{(r_m r_i)}$ is the input resistance and can be obtained from the Y intercept of this plot. When the plot is made on

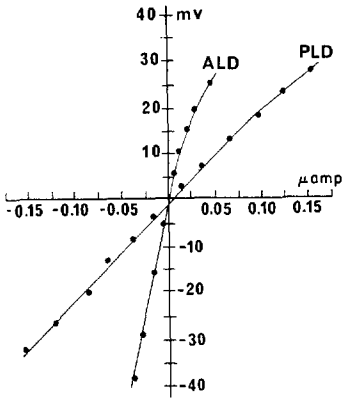

FIGURE 4. Graphic presentation of current-voltage relationship for fibers of the ALD and PLD. Depolarization is plotted upward.

$\log_{10}$ paper, the slope of the line equals $-1/(2.303\ \lambda)$. From the values of the intercept and the slope, r_i and r_m can be calculated.

The specific resistance of the myoplasm, R_i, was estimated by correcting the value determined for the frog at 22°C (230 Ωcm [Katz, 1948]) for the

TABLE I

VALUES OF VARIOUS MEMBRANE CHARACTERISTICS OF TWO TYPES OF MUSCLE FIBERS

Bird	Fiber	*R.P.*	*V/I*	λ	τ	ρ	R_m	C_m
		mv	*MΩ*	*mm*	*msec*	*μ*	*Ωcm²*	*μF/cm²*
A. Anterior latissimus dorsi								
II	1	47	0.940	1.41	42	20	3248	12.9
V	1	51	1.150	2.37	55	23	7843	7.0
	2	50	0.920	2.47	40	26	7453	5.3
	3	47	0.880	2.42	65	26	7091	9.1
	4	50	0.861	1.37	40	27	2983	13.4
VII	1	56	0.563	1.20	15	23	1975	7.5
	2	54	0.640	1.46	15	24	2830	5.3
	3	54	0.512	1.46	17	27	2530	6.7
	4	53	0.517	1.82	24	30	3536	6.7
Mean ±SD		51	0.776	1.78	35	25	4388	8.2
		3	0.225	0.51	18	3	2354	3.0
B. Posterior latissimus dorsi								
I	1	73	0.218	0.64	3.5	27	479	7.3
	2	65	0.175	0.48	2.7	26	279	9.6
	3	60	0.195	0.77	2.0	32	599	3.3
II	1	55	0.338	0.67	4.2	22	638	6.5
III	1	56	0.279	0.91	4.5	29	919	4.8
	2	53	0.152	0.94	3.5	40	711	4.9
	3	56	0.172	0.71	3.2	32	497	6.4
IV	1	55	0.307	0.62	3.5	23	543	6.4
	2	54	0.194	0.62	4.0	28	431	9.2
	3	54	0.218	0.55	4.0	25	381	10.4
VII	1	53	0.549	0.63	5.6	17	742	7.5
	2	55	0.217	0.67	4.0	28	508	7.8
Mean ±SD		57	0.251	0.68	3.7	27	561	7.0
		6	0.110	0.13	0.3	6	174	2.1

difference between the ionic composition of frog Ringer solution and the chicken bathing solution. The NaCl equivalent of the chicken bathing solution is 182 mM while that of frog Ringer solution is 125 mM (Adrian, 1956). If the conductivity, $1/R_i$, is assumed to be directly proportional to the ionic concentration (Hartree and Hill, 1921), the corrected value of R_i for the chicken myoplasm is 160 Ωcm at 22°C. Since the bath was held close to 22°C, no temperature correction is necessary.

The fiber radius and remaining membrane constants can be calcu lated from the following relationships: $\rho = \sqrt{(R_i/\pi r_i)}$ where ρ is fiber radius; $R_m = 2\pi\rho r_m$ where R_m is transverse resistance of a unit area of membrane; and $C_m = \tau_m/R_m$ where C_m is membrane capacitance and τ_m is the time constant of the membrane. τ_m was measured as the time taken for the electrotonic

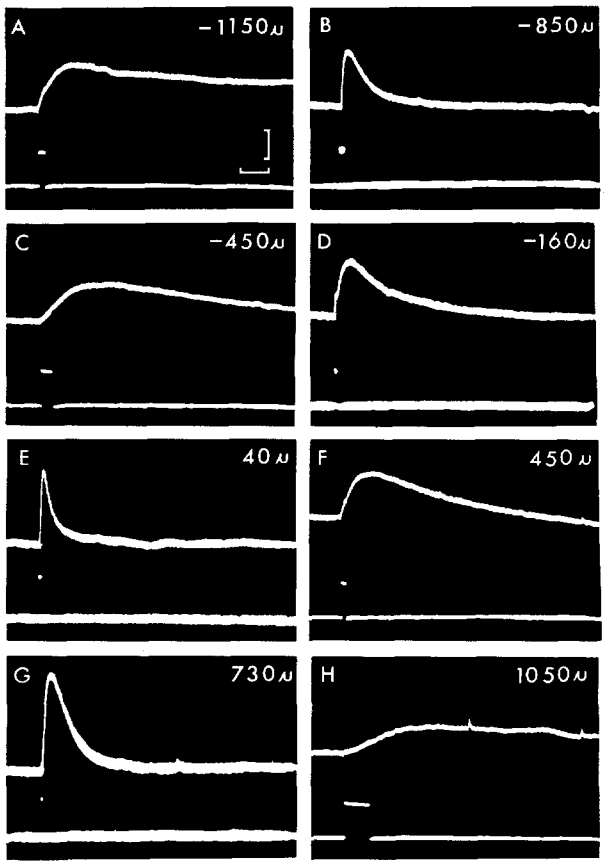

FIGURE 5. Transmembrane responses to iontophoretic application of ACh along the membrane of a fiber from the ALD. Top trace, membrane potential change; bottom trace, current applied to ACh pipette. Vertical scale in A equals 2 mv and 1×10^{-7} amp. Horizontal scale, 500 msec.

potential to fall to 83 % of its maximum steady value during hyperpolarization with the two electrodes as close together as possible ($x/\lambda < 0.1$, Hodgkin and Rushton, 1946).

Records of current-voltage relationships during hyperpolarization of fibers of the ALD and PLD are presented in Fig. 3. Fibers of both muscles exhibited linear current-voltage curves during hyperpolarization and during small de-

grees of depolarization (<10 mv; Fig. 4). The membrane constants were determined using hyperpolarizing pulses.

The membrane constants obtained for 9 fibers of the ALD from 3 birds and 12 fibers of the PLD from 5 birds are shown in Table I. Mean input resistance was markedly higher in fibers of the ALD than in fibers of the PLD. Likewise, the mean length constant of fibers in the ALD was more than twice that of fibers in the PLD, and the mean time constant was approximately 10 times longer in the former than in the latter. The transverse resistance of a unit area of membrane, R_m, was approximately eight times greater in fibers of the ALD than in those of the PLD, although no difference was found be-

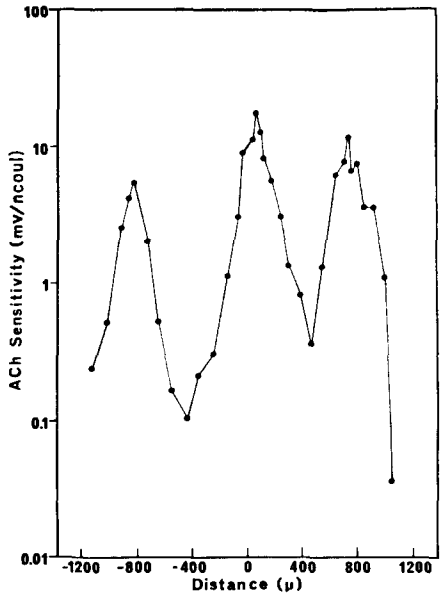

FIGURE 6. ACh sensitivity at various sites along the membrane of a fiber from the ALD. Distances were measured from the recording electrode. Since the length of membrane tested was much shorter than λ, no correction for potential decay with distance was made. Three distinct peaks of ACh sensitivity averaging 790 μ apart were found.

tween fibers from the two muscles in calculated fiber radius or membrane capacitance. The radii of fibers determined from the electrical measurements compare reasonably well with those found from histological measurement. The mean radius obtained by the latter method for 200 fibers from the ALD was 32.8 ± 0.3 μ (SE) while that for 200 fibers from the PLD was 27.8 ± 0.4 μ. These values are similar to those obtained from these muscles by Hess (1961, Plate 1).

Acetylcholine Sensitivity Sensitivity of the fiber membrane to ACh was investigated at various sites using iontophoretic techniques. Fig. 5 illustrates changes in membrane potential produced by this procedure at several sites along a fiber from the ALD. The membrane of fibers of this muscle was sensitive to ACh at most of the sites tested, although the degree of sensitivity varied.

ACh sensitivity was estimated by measuring the number of coulombs passed through the ACh electrode that produce 1 mv decrease in membrane potential and has been plotted as millivolts/nanocoulomb (mv/ncoul) (Miledi 1960 *a*). The results for the fiber shown in Fig. 5, where 33 membrane sites were tested, illustrate three peaks of ACh sensitivity (Fig. 6). In two fibers of the ALD, ACh sensitivity was tested along a length of more than 2 mm, and three peaks of sensitivity were found with an average of 796 μ between peaks. In a total of nine fibers, a sufficient length was tested to show more than one peak of ACh sensitivity. The average distance between peaks was 743 μ.

A total of 18 fibers of the ALD was successfully tested to show at least one peak of sensitivity. The mean of the maximum ACh sensitivity for 28 peaks was 5.2 $\pm$ 4.4 mv/ncoul (Table II).

Miniature end plate potentials (m.e.p.p.'s) could be recorded at almost

TABLE II

ACETYLCHOLINE SENSITIVITY OF THE JUNCTIONAL MEMBRANE OF FIBERS OF THE ALD AND PLD

The gradient of ACh sensitivity is indicated by the distance from the peak of ACh sensitivity to the site of 1/10 peak sensitivity on each side of the peak. Mean ±SD.

	No. of ACh peaks	Maximum ACh sensitivity	Gradient of ACh sensitivity
		mv/ncoul	μ
ALD	28	5.2 ± 4.4	227 ± 75
PLD	7	77 ± 60	142 ± 77

every location along fibers of the ALD as found by Ginsborg (1960 *b*). In one fiber the m.e.p.p. frequency averaged 0.39/sec, and the duration of the m.e.p.p.'s was about 100 msec as measured from the beginning of depolarization to the completion of repolarization. In fibers of the PLD, often no m.e.p.p.'s could be found even after hours of searching. A systematic exploration for m.e.p.p.'s from surface fibers of the PLD indicated that the end-plates were distributed at random. The average m.e.p.p. frequency in two fibers from the PLD was 13/sec, and the duration of the potentials was about 4 msec.

Well-defined ACh sensitivity curves were obtained from the application of ACh at 7–18 sites on a total of 7 end plates in the PLD. A plot of the ACh sensitivity at various sites along the membrane of one such fiber shows that a sharp, symmetrical peak of sensitivity was present (Fig. 7). This type of peak correlates with the rather compact, discrete motor end plates found in this muscle (Hess, 1961). The maximum sensitivity obtained was much greater in the PLD than in the ALD (Table II). No detectable change in the membrane potential was caused by iontophoretic application of large amounts of ACh

at regions where m.e.p.p.'s were not recorded. However, ACh sensitivity was not determined at the myotendious junction, so that a low sensitivity might have been present at this location (Katz and Miledi, 1964). The extrajunctional fiber membrane in the areas examined was thus similar to those of frog and mammalian twitch muscles (Axelsson and Thesleff, 1957; Miledi, 1960 *a*, *b*).

The spatial gradient of ACh sensitivity was significantly different between the fibers of the ALD and PLD (Table II). The gradient was much steeper

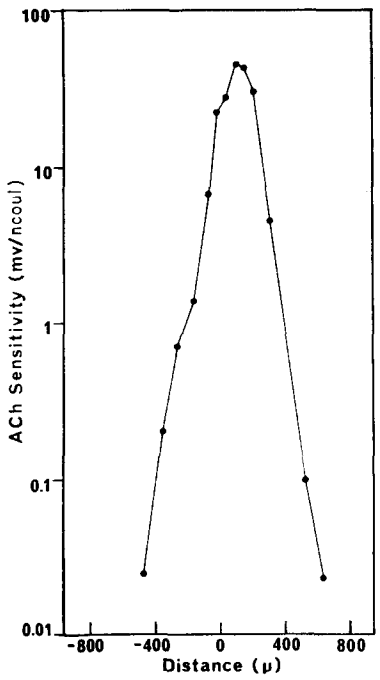

FIGURE 7. ACh sensitivity at various sites along the membrane of a fiber from the PLD. Distances were measured from the recording electrode and no correction was made for the length constant. Detectable transmembrane potential changes were not found when ACh was deposited at extrajunctional sites.

for the PLD than for the ALD. This may be related to dispersion of motor endings since an *en grappe* ending occupies a much larger area of membrane than a single *en plaque* ending (Hess, 1961).

Repetitive doses of ACh were iontophoretically applied to various membrane sites on fibers of the ALD to determine the rate of ACh desensitization (Fig. 8). Application of ACh was continued until the membrane showed a definite degree of desensitization. A rest period was then given and the membrane was tested again. In many cases, the membrane did not return to its original sensitivity after the rest period. Since this may have been caused by movement of the ACh electrode, these results were discarded. When the initial sensitivity was relatively high, the rate of desensitization was low, and even after many ACh pulses the level of sensitivity was high (Fig. 9 A and B).

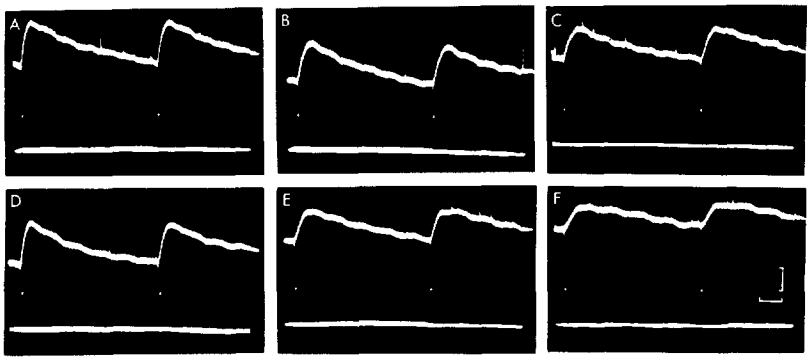

FIGURE 8. Records showing the degree of ACh desensitization of a fiber of the ALD produced by repeated iontophoretic applications of ACh. Pulses were given every 11.6 sec. A, B, and C are not continuous records and were taken prior to resting the fiber while D, E, and F were taken after the rest period. Time between A and B is 23 sec; between B and C, 46 sec; between C and D, rest period of 2 min; between D and E, 23 sec; between E and F, 46 sec. These data are presented in graphic form in C and D of Fig. 9. Initial sensitivity of the fiber at this site was 0.46 mv/ncoul. The fiber gradually desensitized but returned almost to the original level of sensitivity after a 2 min rest period. Vertical scale is 2 mv and 1×10^{-7} amp. Horizontal scale represents 2 sec; pulse duration was 56 msec.

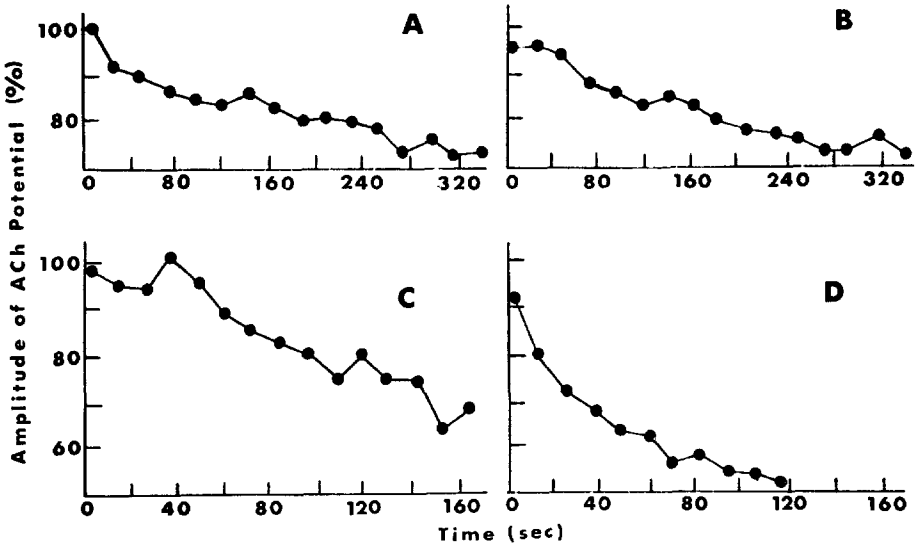

FIGURE 9. Degree of desensitization to ACh on fibers of the ALD. Initial sensitivity in A, 5.2 mv/ncoul and in C, 0.46 mv/ncoul. In A and B, ACh pulse given every 5.6 sec but only every fourth pulse is shown. In C and D, ACh pulse given every 11.6 sec. Rest period is 5 min between A and B; 2 min between C and D.

However, the rate of desensitization was considerably greater at relatively insensitive membrane sites (Fig. 9 C and D). In general, the membrane of fibers of the ALD appeared to be desensitized much more slowly at a given sensitivity level than fibers of the retractor capitis muscle of the tortoise (Levine, 1966).

DISCUSSION

Striking differences were found in the membrane properties of fibers of the slow, multiinnervated ALD and the fast, focally innervated PLD of the chicken. Most notable were the membrane time constant, τ_m, length constant, λ, and transverse resistance of a unit area of membrane, R_m. Since R_m and τ_m were proportionally greater in fibers of the ALD, the membrane capacitance was very similar between these fibers and those in the PLD and was similar to that of fibers of the frog sartorius (del Castillo and Machne, 1953). Fatt (1964) found that the surface membrane capacitance for fibers of the ALD and PLD of the chicken was 2.79 μF/cm^2. If we assume that this value is 39 % of the total capacitance of these fibers (Falk and Fatt, 1964), C_m should equal a value of 7.2 μF/cm^2. This is reasonably close to the value found in the present study by the rectangular pulse method.

Page and Slater (1965) pointed out that the transverse tubular system and sarcoplasmic reticulum come into close apposition in the PLD, while in the fibers of the ALD these two systems are still distinct but are rarely found in close apposition as in the triad. Mayr (1966) recently reported that a fully developed T-system with a great number of triads on the A-I border was present in all the fibers investigated from both the PLD and the ALD, even though the number of triads in the PLD appeared to be greater. If from 61–64 % of the membrane capacitance is due to the expanded membrane of the transverse tubular system (Fatt, 1964; Falk and Fatt, 1964; Freygang et al., 1967; Eisenberg and Gage, 1967), the similar values of C_m in fibers from both the ALD and PLD suggest that a similar transverse tubular system exists in both types of fibers.

The innervation of the ALD is considerably different from that of the PLD. The motor endings in the ALD terminate at regular intervals of about 1000 μ in adult chickens (Hess, 1961) and are of the *en grappe* type which, when stained for cholinesterase, appear as dispersed droplets with a considerable exposed area of the muscle membrane between cholinesterase concentrations (Hess, 1961; Silver, 1963). The multiple peaks of sensitivity recorded for membrane sensitivity to ACh were 743 μ apart. These peaks were most probably associated with the sites of motor endings. The average maximal sensitivity to ACh was considerably lower in the ALD (5 mv/ncoul) than in the PLD (77 mv/ncoul). Whether the low sensitivity in the ALD is repre-

sentative of the postsynaptic "receptor" in the *en grappe* ending is uncertain, since the consistent application of ACh directly to a single component of the ending would be somewhat improbable.

The finding that ALD fibers have both a greater membrane resistance and a greater chemosensitive area than the PLD fibers raises the question of whether these two factors are related. Some evidence that this is the case comes from denervation studies on frog skeletal muscle. Following denervation of twitch muscle fibers in the frog, both the chemosensitive area and the membrane resistance increase (Nicholls, 1956; Miledi, 1960 *a*).

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