Depolarization of the Internal Membrane System in the Activation of Frog Skeletal Muscle

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ABSTRACT "Skinned" muscle fibers, single fibers from the frog semitendinosus muscle in which the sarcolemma had been removed, could be reversibly activated by electrical stimulation. Electrical responsiveness was abolished when the skinned fiber was prepared from a muscle exposed to a cardiac glycoside, and the development of responsiveness was delayed when the muscle was bathed in high potassium solution. The findings were taken as evidence that active sodium-potassium exchange across the internal membranes restored electrical excitability, after the sarcolemma had been removed, by establishing a potential gradient across the internal membranes. In general, the contractions were graded with the strength of the applied current. On occasion, however, "all-or-none" type responses were seen, raising the possibility that the internal membranes were capable of an electrically regenerative response. Activation could also be produced by an elevation of the intracellular chloride ion concentration or a decrease in the intracellular potassium ion concentration, suggesting that depolarization of some element of the internal membrane system, that is, a decrease in the potential of the lumen of the internal membrane system relative to the potential of the myofibrillar space, was responsible for activation in these experiments. The distribution of both the electrically induced contractions and those produced by changes in the intracellular ion concentrations indicated that the responsive element of the internal membrane system was electrically continuous over many sarcomeres.

Csapo (1959) and Natori and Isojima (1962) have reported that single fibers from amphibian muscle could still be activated by electrical stimulation following removal of the surface membrane. It seemed possible that current flow across some component of the internal membrane system could account for this phenomenon and the experiments described in this paper are an attempt to examine this question. Preliminary accounts of these results have been published (Costantin and Podolsky, 1965, 1966).
METHODS

Experimental Preparation

All experiments were performed on the Natori skinned fiber preparation (Natori, 1954) at room temperature (about 23°C). A bundle of 6-10 fibers was removed from a frog semitendinosus muscle, blotted lightly, and covered with paraffin oil on a microscope slide. A short segment of one fiber was dissected free and the sarcolemma manually removed from about half its length. The procedure involved dissecting the sarcolemma and a superficial layer of myofibrils from the bulk of the fiber in one region and teasing this along the fiber until a length of about 1 mm had been skinned; the sarcolemma can be removed in this way much as a sleeve is rolled up. The larger fibers within the semitendinosus (diameter, 80-100 μ) were chosen for skinning. Since the layer of myofibrils which was removed with the sarcolemma was very thin, the diameter of the skinned region was usually at least 90% of the diameter of the fiber before skinning. Following dissection, the preparation was placed on a microscope stage, permitting simultaneous visual observation and cinemographic recording.

The skinned fiber preparation remains relaxed following removal of the sarcolemma, responding to local application of calcium ion with a local twitch-like contraction (Podolsky and Hubert, 1961). About 40-60 min following dissection, however, the skinned region “collapses,” that is, an irreversible contraction develops at one site and slowly spreads to involve the entire skinned region. All experiments were stopped at the first sign of “collapse.”

In most experiments, the preparation was illuminated by ordinary light with the condenser aperture stopped down until striations were readily visible, and was observed with a 10 × Zeiss objective (NA = 0.22) or a 40 × Bausch & Lomb oil-immersion objective (NA = 1.00) which made contact with the paraffin oil bathing the preparation. When better resolution of single contracting sarcomeres was required, the specimen was observed in an inverted microscope equipped with a Unitron 100 × phase contrast objective (NA = 1.25).

Stimulation Procedure

ELECTRICAL STIMULATION When the preparation was to be stimulated electrically, the skinned fiber was prepared from the central segment of the isolated fiber bundle, leaving two large bundles of muscle tissue at either end of the skinned region in which silver wire electrodes could be positioned. This arrangement provided a large surface area of electrode contact relatively far from the skinned portion of the fiber so that the irreversible and presumably unphysiological contractures seen at the site of contact between electrode and preparation (Natori and Isojima, 1962) could be avoided in the skinned portion. A photomicrograph of this arrangement is shown in Fig. 1.

Current strength was monitored as the voltage drop across a 100 KΩ resistor. Direct currents larger than about 15 μA which were maintained for longer than 2–3 sec induced changes in the gross appearance of the preparation, the main effect
FIGURE 1. Electrical activation of skinned muscle fiber. A, low power photomicrograph of experimental preparation. Single fiber segment isolated between two multifiber bundles; the sarcolemma has been removed from the junction with the muscle bundle on the right to the point in the center of the preparation indicated by the black arrow. The silver wire electrodes in the two bundles of muscle tissue at the ends of the single fiber are indicated by white arrows. The electrode in the muscle bundle on the right is not clearly seen. B and C, higher magnification of the region outlined in panel A. B, before stimulation, C, peak of contraction during 1 sec dc pulse of 5 μA. D and E, higher magnification of the region outlined in panels B and C. D, before stimulation, E, peak of contraction during 500 msec dc pulse of 10 μA. The contracting regions are indicated by arrows. The magnitude of the contraction to 5 μA had decreased between C and E.
being an increase in the granularity of the sarcoplasm. When more than four or five stimuli of such high current strength were applied, the entire preparation usually "collapsed;" accordingly, electrical stimulation was routinely limited to currents below 15 μA for durations less than 1 sec.

**DROPLET STIMULATION** Small volumes of solution were placed on the surface of the skinned fiber by means of a micropipette. Pipettes which had been drawn on a conventional micropipette puller and coated with a silicone preparation (Desicote; Beckman Instruments, Inc., Fullerton, Cal.) were broken to the desired tip size (2–10 μ OD) by gentle stroking along a ground glass stopper. It was found that breakage was most readily controlled when the pipette had a long gentle taper. The pipette tip was filled by suction with the solution to be applied to the fiber, and the shaft was connected to a closed, fluid-filled system in which pressure could be in-

<table>
<thead>
<tr>
<th>Solution</th>
<th>K⁺</th>
<th>Na⁺</th>
<th>Ca²⁺</th>
<th>Sucrose</th>
<th>Cl⁻</th>
<th>SO₄²⁻</th>
</tr>
</thead>
<tbody>
<tr>
<td>A*</td>
<td>2.5</td>
<td>11.5</td>
<td>1.8</td>
<td>207</td>
<td>17.6</td>
<td>—</td>
</tr>
<tr>
<td>B*</td>
<td>2.5</td>
<td>115.5</td>
<td>1.8</td>
<td>—</td>
<td>121.6</td>
<td>—</td>
</tr>
<tr>
<td>C‡</td>
<td>2.5</td>
<td>77.5</td>
<td>8.0</td>
<td>113</td>
<td>—</td>
<td>48</td>
</tr>
<tr>
<td>D†</td>
<td>80.0</td>
<td>—</td>
<td>8.0</td>
<td>113</td>
<td>—</td>
<td>48</td>
</tr>
</tbody>
</table>

* Buffered at pH 7.0 with 3 mM sodium orthophosphate.
‡ Buffered at pH 7.0 with 1.5 mM sodium orthophosphate.

creased by advancing a syringe plunger with a micrometer. The pipette was then mounted in a micromanipulator, and the tip inserted into the oil layer above the fiber. Pressure was applied until a droplet of the desired size was formed, and the droplet was then lowered to the fiber surface with the micromanipulator.

Most droplets did not remain at the very tip of the pipette but rather attached themselves to one side of the pipette shaft a few microns from the tip. The silicone coating on the pipette prevented the droplet from wetting the pipette and spreading along it, so that the droplets usually remained nearly spherical in shape. Drop diameters were measured at right angles to the axis of the micropipette with an ocular micrometer (estimated accuracy of measurement ±5 μ), and volumes were calculated on the assumption that the droplets were spherical.

**Solutions**

The semitendinosus muscle from which skinned fibers were prepared was mounted in a dissecting chamber filled with one of the solutions in Table I. In most experiments solution A was used; unless otherwise noted, similar results were obtained when solution B or C was employed. When the effect of strophanthidin was to be investigated, the strophanthidin (Sigma Chemical Co., St. Louis, Mo.), dissolved in an appropriate volume of absolute ethanol, was added to solution C in desired amounts (Horowicz and Gerber, 1965). The final concentration of ethanol was 1
ml/liter of solution and a similar amount of ethanol was added to the control solution in this experiment.

All solutions applied as droplets to the surface of the skinned fiber were 140 mM in concentration of a uni-univalent salt. K or Na propionate was made by titrating propionic acid to pH 7 with the appropriate base. K methylsulfate salt was obtained from Eastman Organic Chemicals, Rochester, N. Y. and the solution was filtered before use. Solutions of Tris salts were made by titrating tris(hydroxymethyl)aminomethane (Trizma base—Sigma Chemical Co.) to pH 7 with either HCl or propionic acid.

RESULTS

Electrical Stimulation

DISTRIBUTION OF RESPONSE Passage of direct current pulses (less than 15 μA and 200–1000 msec in duration) between the electrodes resulted in reversible contractions in the skinned portion of the fiber. The contractions involved only a very small part of the entire 1 mm length of skinned fiber; moreover, only a few of the sarcomeres within this responsive region actually contracted, usually in groups of two–five adjacent sarcomeres with apparently relaxed sarcomeres between (Fig. 1). Maximum shortening developed within about 200 msec and usually persisted for the duration of the stimulus. When stimuli of longer duration were applied, spontaneous relaxation occurred during stimulation, and contractions could not be maintained for longer than 1–2 sec. If the direction of current flow was reversed, variable results were obtained. In many instances, no response was elicited until the current was increased, while in others, a more vigorous response resulted from reversal of electrode polarity. Although the same general region of fiber usually contracted with reversed current flow, the distribution of contracting sarcomeres within this region was quite different and bore no apparent relation to the initial distribution.

GRADATION OF RESPONSE An increase in stimulus strength usually produced contractions in other regions of the skinned fiber and also recruited more sarcomeres in the initially responsive region. It was not possible, however, even with currents of 100 μA, to cause the entire skinned region to contract. Since the extracellular current path in the unskinned region consisted only of a 2–3 μ layer of fluid surrounding the fiber, appreciable currents would be expected to flow within the unskinned segment during stimulation. Nevertheless similar responses were never seen in the unskinned region.

It was possible to elicit rather different contractions in the unskinned segment, although currents greater than 15 μA were usually required. These contractions developed slowly during stimulation, often not beginning until about 500 msec after the onset of current flow. They began with shortening of two to three sarcomeres...
FIGURE 2. Gradation of electrical response with increasing current. A, segment of skinned muscle fiber before stimulation. B-F, peak of contraction elicited by 200 msec
at the very edge of the fiber, and, when stimulation was maintained for 2-3 sec, the contraction spread radially from the initial site for up to 20 μ. Apparently complete relaxation occurred when current flow was stopped.

In many instances, disruption of the sarcolemma was evident at the site where these contractions originated; such damage might be expected to provide a low resistance current path through the membrane and result in an abnormally high current density in the damaged region. When deliberate injury to a small region of an unskinned fiber segment was produced with a micropipette, the subsequent application of 15 μa direct current to the preparation resulted in a contraction at the site of injury identical to those described above. No further study was undertaken of these responses.

Although repeated stimulation produced a progressive decrease and eventual disappearance of the contractile response, some preparations maintained apparently stable responses for as long as 10 min with intermittent stimulation, and an attempt was made in these preparations to examine the mode of spread of the response with increasing current strengths. Fig. 2 shows cinemicrographs from one of these experiments. As can be seen, the extent of contraction increased by the recruitment of adjacent sarcomeres longitudinally and adjacent myofibrils laterally; thus the actively contracting region spread rather symmetrically in all directions. No obvious preferential spread was found which might be attributable to the polarity of the applied current.

“Break” responses Brief localized contractions which followed cessation of dc pulses of 200 msec or longer were readily obtained in both the skinned and unskinned portions of the preparation, but were not studied in detail.

Current threshold In most experiments, 200 msec pulses were employed for stimulation, and the lowest current that elicited contraction was 1.5 μa in a fiber of ~ 100 μ diameter, a mean longitudinal current density of about 20 ma/cm². Thresholds were variable, however, and usually a current of 4-5 μa, equivalent to a longitudinal field of ~ 10 v/cm (see Appendix), was needed to elicit visible contraction. About 10% of all preparations tested were totally unresponsive to currents as large as 15 μa (the highest current strength routinely employed in this study); unresponsive preparations were obtained more frequently when the dissection was hindered by the presence of large amounts of connective tissue within the muscle.

dc pulse of 3.0, 3.5, 4.0, 5.0, and 5.5 μa respectively. The vertical lines at the top of the panels mark the same sarcomeres in each instance. The direction of positive current for all stimuli is indicated by the arrow in panel A. Phase contrast microscopy (Unitron 100 X oil immersion lens, NA = 1.25). Photographed at 15 frames/sec. Grid spacing, 10 μ.
STRENGTH-DURATION CURVE. The progressive decrease in responsiveness with repeated stimulation prevented the precise determination on a single preparation of the variation of threshold current with stimulus duration. Instead, a composite strength-duration curve was constructed by exploration of a limited range of pulse durations in each preparation. In practice, the threshold current was first determined for a 200 msec square wave stimulus, and then for three additional pulse durations in a given range (2-10 msec, 10-50 msec, or 50-300 msec). The threshold for the 200 msec stimulus was redetermined and the entire sequence was repeated one or more times. The experiment was terminated when an increase in threshold current for the 200 msec stimulus of greater than 30% was noted; the preparation generally became totally unresponsive to electrical stimulation within 2-3 min after this had occurred. Two satisfactory preparations were obtained in each range. The average threshold current at each stimulus duration was normalized to the value for a 200 msec stimulus in the same preparation, and the normalized values were employed for comparison between preparations. The results are plotted in Fig. 3. It will be seen that the threshold current reaches twice its limiting value when the stimulus duration is about 10 msec.

DC VS. AC STIMULATION. Contractions were readily elicited in the skinned region with sine wave stimulation; these were somewhat larger than those
obtained with DC pulses of 200–1000 msec duration in that they frequently appeared to involve up to 10 adjacent sarcomeres, but again it was not possible to activate more than a small fraction of the entire skinned region. A progressive increase in threshold current was invariably found as the frequency of stimulation was increased over the range 50–1000 cps. The mean threshold currents from five preparations are given in Table II.

**Evidence for a Resting Potential across the Internal Membrane System**

As noted above, a longitudinal DC field of ~10 v/cm maintained for longer than 100 msec was usually required for activation in the present experiments. This is much larger than the field established within the intact fiber by the action potential, which Sten-Knudsen (1960) has estimated to be about 3 v/cm for about 0.3 msec, and the possibility must be considered that this relatively high field strength produces structural damage within the fiber substance, perhaps to some element of the internal membrane system, with a resultant leakage of calcium into the myofilament space. However, the spotty distribution of responses in the skinned region and the total absence of similar responses in the unskinned region of the fiber argue against nonspecific structural damage to the fiber by the applied field. Moreover, there is reason to believe that the potential changes which should arise across the walls of the internal membrane system with the present stimuli are not excessive (see Discussion). The slow contractions seen at sites of surface membrane damage in the unskinned region (as described above) probably were a result of structural damage to the internal membranes and in fact could usually be induced only with very strong stimuli which, when repeated a few times, resulted in progressive collapse of the entire preparation.

Electrical activation in intact muscle is abolished when the membrane potential falls below 20–30 mv (Jenerick and Gerard, 1953). This is presumably the situation in the unskinned region of the preparation, since the

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**Table II**

**Threshold Current at Different Frequencies of Stimulation**

<table>
<thead>
<tr>
<th>Mode of stimulation</th>
<th>Relative current strength*</th>
</tr>
</thead>
<tbody>
<tr>
<td>DC</td>
<td>1</td>
</tr>
<tr>
<td>50 cps sine wave</td>
<td>1.27±0.61</td>
</tr>
<tr>
<td>500 cps sine wave</td>
<td>2.06±0.96</td>
</tr>
<tr>
<td>1000 cps sine wave</td>
<td>3.05±1.80</td>
</tr>
</tbody>
</table>

*Threshold currents were normalized for comparison among different preparations. AC thresholds represent root mean square values ± 1 SD. The mean DC threshold for the five preparations was 4.5 μA.
isolation of a short length of a single fiber might be expected to damage the surface membrane, permitting the leakage of some intracellular potassium into the thin layer of fluid surrounding the fiber in oil. The excitability of the skinned segment, therefore, implies that removal of the surface membrane permits the restoration of excitability in some way. One possible mechanism for this restorative process is that the transverse tubules (T system) can reseal following their disruption from the surface membrane, and that the operation of a metabolically supported sodium pump within the T system is then capable of decreasing the potassium concentration within the tubular lumen. The establishment of a potassium diffusion gradient would result in a resting potential across the internal membranes, with the lumen of the internal membrane system\(^1\) positive with respect to the myofilament space.

**Responsiveness of digitalized muscle** This hypothesis for the responsiveness of the skinned fiber to electrical stimulation predicts that inhibition of active sodium transport, by preventing the establishment of a potassium gradient across the walls of the internal membrane system, should abolish excitability, and this prediction was directly examined by studying the responsiveness of skinned fibers prepared from digitalized muscle. Muscles were bathed in solution C containing 1 or 2 × 10\(^{-6}\) \(M\) strophanthidin, concentrations which have been shown (Horowicz and Gerber, 1965) to inhibit active sodium transport in frog semitendinosus muscle. The experimental procedure for testing responsiveness was as follows: Two skinned fiber preparations from a muscle bathed in solution C were tested by electrical stimulation. The solution bathing the muscle was then changed to solution C with added strophanthidin, and five additional skinned fiber preparations, dissected 15–40 min following transfer to the glycoside solution, were tested. The bathing medium was then changed to the original glycoside-free solution, and five additional skinned fiber preparations, dissected 15–60 min following the change of solution, were tested. Finally, three preparations, dissected after the muscle had been in the recovery solution for longer than 90 min, were tested. The test for electrical responsiveness consisted of applying dc pulses of progressively increasing current strength up to 15 \(\mu A\) to the skinned fiber. The initiation and duration of the test current were controlled manually, and pulse duration ranged from 200–1000 msec. This arrangement permitted the observer to distinguish readily between a “break” response and the direct response to a maintained stimulus.

The results with the three muscles examined are given in Table III. As can be seen, exposure to strophanthidin reversibly abolished the responsive-

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\(^1\)The question whether a polarization of the T tubules is transmitted to the membranes of the sarcoplasmic reticulum (SR) cannot be answered with certainty and, for the present, the general term “internal membrane system” will be used in dealing with potential changes across either or both of these elements. The problem is treated further in the Discussion.
Depolarization of Internal Membranes

The recovery following glycoside exposure was apparently complete; the mean threshold current in the fibers tested before treatment was 3.6 μA while it was 3.1 μA in those fibers dissected from the muscle 90 min or longer following exposure to strophanthidin.

**ELECTRICAL RESPONSIVENESS AFTER EXPOSURE TO HIGH K SOLUTIONS**

The evidence that an active Na-K exchange across the internal membranes restored excitability in the skinned fiber suggested that electrically excitable preparations might be obtainable from muscles which had been depolarized before dissection. To examine this possibility, skinned fibers prepared from a muscle bathed in a solution containing 80 mM K (solution D) were studied;

| TABLE III |
| Effect of Strophanthidin on the Electrical Responsiveness of Skinned Muscle Fibers |
| The numbers in each column represent the number of responsive preparations/number of preparations examined. |
| Before strophanthidin treatment | During strophanthidin treatment | 15-60 min after strophanthidin treatment | >90 min after strophanthidin treatment |
| Muscle 1* | 2/2 | 0/5 | 1/5 | 3/3 |
| Muscle 2‡ | 2/2 | 0/5 | 2/5 | 3/3 |
| Muscle 3‡ | 2/2 | 0/5 | 0/5 | 3/3 |

* Treated with 2 × 10⁻⁵ M strophanthidin.
‡ Treated with 1 × 10⁻⁴ M strophanthidin.

Application of the test current was controlled manually so that pulse durations were 200–1000 msec. Although the membrane potential of the intact fiber in this solution should be -15 mV (Hodgkin and Horowicz, 1959), skinned fiber preparations responsive to currents of 2–7 μA could be readily obtained.

Although in general the responsiveness of skinned fibers prepared from media containing 2.5 mM K decreased with time, repeated testing with the same stimulus over the first 1–2 min following dissection occasionally produced progressively larger responses. This effect was slight, however, and it was difficult to be certain that an actual delay in the development of responsiveness was occurring. In the preparations dissected from the solution containing 80 mM K, however, a definite delay in the development of responsiveness was seen. The entire dissection required about 5 min, while the time between the removal of the surface membrane and the beginning of electrical stimulation was 2–3 min. Of the nine preparations tested, seven were initially unresponsive to the maximum currents employed (15 μA); a positive response
was obtained, however, when the preparations were tested 2–7 min later. All nine preparations showed a decreasing threshold during repeated stimulation.

It is of interest to compare the delay in development of excitability in high potassium solutions in the present experiments with the time required for an active sodium transport process to clear the tubules of potassium. Horowicz and Gerber (1965) give 3.7 pm per cm² of surface membrane per sec for the resting Na efflux in a single frog semitendinosus fiber of 70 μ diameter and attribute 77% of this efflux to a strophanthidin-sensitive sodium pump. Assuming that the pump capacity is equally distributed over the outer surface membrane and the transverse tubular membranes, the total membrane area available for pumping in a 70 μ fiber is 5.9 cm² per cm² of muscle surface (Peachey, 1965); the pump-supported Na efflux would then be 0.48 pm per cm² of membrane per sec. Assuming a 1:1 Na-K exchange and neglecting the passive diffusion of K, such a pump would clear a tubule 500 A in diameter of 80 mM K in about 3.5 min. The stimulation of active sodium transport by membrane depolarization would be expected to shorten this by a factor of three or more (Horowicz and Gerber, 1965) while the back diffusion of K from the myofilament space would prolong it; nevertheless it is clear that the observed delay is of the same order of magnitude as predicted from the hypothesis that it arises from a metabolically supported Na-K exchange across the T tubule membrane. Similar considerations with the assumption that the sodium pump is distributed equally over the SR and T tubule membranes result in a pumping rate of 0.027 pm per cm² of membrane per sec and a predicted recovery time of more than 200 min.

**Ionic Activation**

The potential gradient across a biological membrane can be altered, not only by the application of electric current, but also by appropriate changes in the ionic environment of the membrane. Since the surface membrane of the skinned fiber is removed under paraffin oil, the composition of the medium bathing the internal membrane system remains essentially that of normal sarcoplasm, that is, it is high in potassium and low in permeant anions. An increase in the concentration of a permeant anion will tend to drive the potential of the myofibrillar space positive with respect to the lumen of the internal membrane system; the magnitude of this potential change would, of course, depend both on the relative permeability of the internal membranes to all ions in the medium and on the concentration gradient of each ion.

A transient alteration of the internal environment of a segment of the skinned fiber preparation is readily produced by the addition of a droplet to the fiber surface, and when droplets of 140 mM KCl, 60–150 μ in diameter, were applied to the skinned region, a reversible contraction lasting 1–2 sec was seen in about 50% of the trials. Application of a second chloride droplet to the same region resulted in either no response or a markedly reduced response. An example of a chloride-induced contraction is shown in Fig. 4.
FIGURE 4. Response of skinned muscle fiber to KCl droplet. A, segment of skinned fiber at moment of contact of 0.3 ml droplet of 140 mM KCl with surface of skinned fiber. B-F, 0.5, 1, 2, 3, and 4 sec following droplet contact. Note the asymmetrical distribution of the contraction about the region of application of the chloride solution. Grid spacing, 10 μ.
CHARACTERISTICS OF THE RESPONSE. The distribution of the chloride-induced contraction appeared to be determined by the inherent responsiveness of the fiber itself. In the experiment shown in Fig. 4, for example, the applied droplet remained well circumscribed, and, presumably, diffusion into the bulk of the fiber produced a symmetrical elevation of the chloride ion concentration about the site of application; nevertheless, a markedly asymmetrical contraction resulted. In some cases, the chloride-induced response arose up to 100 $\mu$ from the site of droplet application, again suggesting that the inherent responsiveness of the fiber itself determined the distribution of the resultant contraction. Contractions ranged in extent from involvement of three to four adjacent sarcomeres over one-third of the fiber diameter to apparently complete activation of the fiber over a 300–400 $\mu$ length of the skinned segment.

RESPONSE PROPAGATION. When the chloride-induced response involved more than 100 $\mu$ of the fiber length, it was possible to estimate the speed of propagation of the response along the fiber by measuring the progression of the leading edge of the contraction from the site of droplet application with time. No attempt was made to correct for the actual shortening of the contracting region. The speed of propagation varied greatly, even within the same fiber, but in general was less than 50 $\mu$/sec. This rate of spread of contraction is compatible with the time required for free diffusion from the applied droplet to raise the chloride ion concentration within the fiber substance, especially when the variable contribution to the spread of added chloride by bulk flow of fluid from the droplet along the fiber surface is considered. When the response arose at some distance from the site of droplet application, latencies of up to 1 sec between the time of droplet application and initiation of contraction were frequently seen, and presumably reflected the time required for chloride to diffuse from the droplet into a responsive region of the fiber.

In a few instances, the velocity of propagation seemed much too rapid to be attributable to simple diffusion of chloride from the droplet. In one preparation, for example, where the leading edge of the contraction had extended to 150 $\mu$ from the edge of the applied droplet over a 5 sec period, the contraction involved the adjacent 75 $\mu$ of the fiber within the next 100–200 msec. Such observations suggested that the skinned fiber possessed an intrinsic mechanism for the longitudinal propagation of contraction; a more unequivocal demonstration of this property was seen in spontaneously contracting fibers (see Spontaneous Contractions).

COMPARISON OF ELECTRICAL AND CHLORIDE ACTIVATION. The activation of the skinned fiber by application of KCl droplets resembled that obtained by electrical stimulation in that both stimuli were able to activate only a
small percentage of the entire skinned region. When the two modes of stimulation were compared in the same preparation, the application of a chloride droplet to an electrically responsive region usually resulted in contraction. In general, the chloride-induced response was rather larger than the electrically induced one; on occasion, however, responses very similar to electrically induced ones could be produced by chloride application (Costantin and Podolsky, 1965, Fig. 4).

Application of large chloride drops (> 100 µ diameter) to an electrically responsive region invariably abolished the response to electrical stimulation while smaller droplets produced a marked reduction in or transient (10–30 sec) disappearance of the electrical response.

**Anionic Specificity** In examining the effect of different anions on the skinned fiber, the variability in responsiveness from one preparation to another made advisable a direct comparison, within the same preparation, of KCl with the solution to be tested. Accordingly, alternate droplets of KCl and the test solution were applied every 250 µ along the skinned region of a given fiber; this usually permitted the application of four droplets on a single preparation. Droplets were generally 100–125 µ in diameter.

By analogy with the surface membrane (Hutter and Noble, 1960), it might be expected that the internal membrane system would be impermeable to organic anions such as methylsulfate and propionate, and thus that K methylsulfate or K propionate would have no effect on the skinned fiber. This expectation was confirmed, the application of 140 mM solutions of these salts failing to produce contraction in the skinned fiber preparation. KBr, on the other hand, resembled KCl in its ability to activate the skinned fiber, while the substitution of Tris or sodium for potassium did not inhibit responsiveness. It was also possible to elicit contractions with 70 mM KCl solutions while 35 mM KCl seemed ineffective. These results are summarized in Table IV.

**Evidence for Depolarization of the Internal Membrane System As a Factor in Activation**

The addition of chloride ion to the myofilament space can be expected not only to depolarize the internal membrane system but also to produce a secondary flux of potassium ion from the myofibrillar space into the lumen of the internal membrane system. It has been postulated (Freygang, 1965) that such a flux of potassium can serve as a specific trigger for calcium release in the normal activation process, while an alternative view (Ohnishi and Ebashi, 1964) has been that depolarization per se results in calcium release.

The application, to the surface of the skinned fiber, of a droplet of a potassium-free solution such as isotonic Tris propionate would be expected to decrease the potassium concentration of the myofibrillar space and therefore...
to alter the potential across the internal membrane system in the same sense as would an increase in the chloride concentration. The direction of the net potassium flux in the two situations would be different, however, a decrease in myofibrillar potassium resulting in a potassium flux from the internal mem-

### Table IV

**ACTIVATION OF THE SKINNED FIBER BY DROPLET APPLICATION**

<table>
<thead>
<tr>
<th>Composition of droplet solution</th>
<th>No. of fibers tested</th>
<th>No. of responsive fibers</th>
<th>No. of drops eliciting contraction per total drops applied to responsive fibers</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. 0.14 M KCl</td>
<td>51</td>
<td>23</td>
<td>31/43 0/43</td>
</tr>
<tr>
<td>B. 0.14 M K methylsulfate</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>A. 0.14 M KCl</td>
<td>28</td>
<td>21</td>
<td>32/43 0/43</td>
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<tr>
<td>B. 0.14 M K propionate</td>
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<td></td>
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<tr>
<td>A. 0.14 M KCl</td>
<td>10</td>
<td>10</td>
<td>10/18 13/18</td>
</tr>
<tr>
<td>B. 0.14 M KBr</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>A. 0.14 M KCl</td>
<td>17</td>
<td>17</td>
<td>16/30 24/30</td>
</tr>
<tr>
<td>B. 0.14 M Tris Cl</td>
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<td></td>
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<tr>
<td>A. 0.14 M KCl</td>
<td>17</td>
<td>14</td>
<td>16/26 13/26</td>
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<td>A. 0.14 M KCl</td>
<td>13</td>
<td>9</td>
<td>14/19 10/19</td>
</tr>
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<td>B. 0.035 M KCl + 0.105 M K propionate</td>
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<td>34</td>
<td>22</td>
<td>22/43 8/43</td>
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<td>B. 0.14 M Na propionate</td>
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<td>9</td>
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<td>B. 0.14 M Na propionate</td>
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Preparations were dissected from muscles bathed in solution A (Table I).

brane system, so that the effectiveness of Tris propionate droplets in eliciting contraction should provide a direct test of these two alternatives. These experiments were performed only on preparations dissected from a muscle bathed in a low chloride Ringer solution (solution A); the intracellular chloride concentration in such preparations would initially be very low (Hodgkin and Horowicz, 1959), and further dilution of the intracellular chloride by
the added droplet of Tris propionate should have little effect on the membrane potential. As can be seen in Table IV, it was indeed possible to activate the skinned fiber by the addition of droplets of 0.14 M Tris propionate, and it would seem, then, that depolarization per se is a primary step in activation in these experiments.

The frequency of positive responses with Tris propionate droplets was somewhat less than that found with KCl droplets, and the responses elicited were less extensive than many of those seen with KCl application, the contraction usually being confined to a region within 50 μ of the edge of the applied drop. This difference in the effectiveness of KCl and Tris propionate application is to be expected from considerations of the concentration changes which can be achieved with the two solutions. If, for example, a droplet 100 μ in diameter were assumed to equilibrate with a 200 μ length of fiber 100 μ in diameter, the internal potassium concentration would decrease only 25% with Tris propionate application while, assuming a normal intracellular chloride of about 2.5 mM, addition of 0.14 M KCl would increase the chloride concentration 15-fold.

Although the presence of two distinct membrane-limited spaces in the muscle fiber makes detailed analysis of the effect of KCl or Tris propionate application highly speculative, it is of interest to consider just one possibility as an example of the complexities which may arise. If the T tubule were assumed to be cation-permselective and the SR anion-permselective, Tris propionate droplets would depolarize the T system while KCl would selectively depolarize the SR. Electrical coupling between the two systems, at the SR-T junction, for example, would result in current flow in opposite directions with the two interventions, and, in this instance also, the common result of the two interventions would appear to be membrane depolarization.

Somewhat surprisingly, Na propionate was almost totally ineffective in eliciting contraction when compared to KCl. The apparent difference between Tris propionate and Na propionate was confirmed by a direct comparison between the two solutions (see Table IV). Although this ineffectiveness could reflect an appreciable permeability of the internal membrane system to sodium, the prior demonstration of an active sodium transport process within the internal membrane system offers an alternative possibility. It has been suggested (Kernan, 1962) that active sodium transport in muscle can be "electrogenic," that is, the displacement of sodium across the membrane can occur without the accompanying movement of other ions, and this net charge transport results in a membrane potential in excess of that produced by passive ionic gradients. In the present experiments in which the addition of sodium propionate elevates the sodium concentration in the myofibrillar space, an electrogenic transfer of sodium into the lumen of the
FIGURE 5. A, B, C, spontaneous contraction in skinned fiber. A, segment of skinned fiber before contraction. B, about 35 msec following onset of contraction. C, maximum contraction about 200 msec following onset. Note that the contraction appears to have begun simultaneously over the entire region between the two arrows in panel B, and shortening progressed without obvious spread of the contraction along the fiber (panel C). The irregular shadow to the right of center in all three panels is a dust particle in
internal membrane system could serve to counteract the change in membrane potential resulting from dilution of myofibrillar potassium.

**Spontaneous Contractions**

Spontaneous contractions, involving 3–20 successive sarcomeres and from one-third to the entire fiber diameter, were noted in about 5% of the preparations studied. The incidence varied greatly from one muscle to another, and on rare occasions more than half the fibers dissected from a given muscle were spontaneously active. In general, the duration of the contraction-relaxation cycle was about 1–3 sec and at least two to three cycles were seen. A few preparations gave apparently periodic contractions, and in two fibers these could be timed over at least 10 successive cycles; in one, the contraction recurred every 23 sec, and, in the other, every 2.5 sec. One of the contractions in the latter fiber is shown in Fig. 5 (A–C). Successive contractions usually grew progressively weaker, involving progressively fewer sarcomeres; all spontaneous activity disappeared within about 10 min after the fiber had been skinned.

While most spontaneous contractions appeared to begin simultaneously over the entire contracting region (Fig. 5, A–C), on rare occasions propagation of the contraction for 100–200 μ along the fiber was observed. An example of a spontaneous response which propagated for a short distance along the skinned fiber is shown in Fig. 5 (D–F).

**DISCUSSION**

**Characterization of the Element of the Internal Membrane System Responsible for Activation**

The present experiments are consistent with the hypothesis that activation can be produced in the skinned muscle fiber by depolarization of some element of the internal membrane system. Since the local stimulation experiments of A. F. Huxley and Taylor (1958) provide rather direct evidence for the participation of the T system in excitation-contraction coupling, the question naturally arises as to whether depolarization of the T system alone is adequate to explain the present results or whether a potential change across the SR must also be postulated.

**Permeability of the Responsive Element** As discussed previously, activation of the skinned fiber by elevation of the intracellular chloride can...
be taken as evidence for depolarization of a chloride-permeable membrane within the skinned fiber. Since the T system appears to be relatively impermeable to chloride ion (Hodgkin and Horowicz, 1959), this would seem to suggest that depolarization of the SR can lead to activation of the skinned fiber. If the resting potential across the T tubule in the skinned fiber were rather close to threshold, however, the large chloride gradient produced by application of 140 mM chloride might result in activation even with a relatively low ratio of chloride to potassium permeability.

LONGITUDINAL EXTENT OF THE RESPONSIVE ELEMENT The most striking characteristic of electrically induced contractions in the skinned fiber is that they are not localized to a single sarcomere but rather involve a variable number of adjacent sarcomeres over a large part of the diameter of the fiber (see Figs. 1 and 2). This distribution of responsiveness suggests that the responsive elements of the internal membrane system are coupled in some way over relatively large regions. The activation by chloride application of many contiguous sarcomeres, rather than of single sarcomeres distributed randomly through the skinned fiber, supports this conclusion, as does the distribution of the spontaneous contractions seen in Fig. 5.

Both the SR and T system appear to be continuous transversely for most of the fiber diameter (Peachey, 1965), but the mode of coupling from one sarcomere to the next in the absence of the surface membrane is not clear. Longitudinal extensions of the T tubules have been described (H. E. Huxley, 1964) and the possibility exists that they might form a continuous network through the bulk of the fiber. Such a model of the T system, however, would be difficult to reconcile with the localization of contraction to a single sarcomere when local surface depolarization is produced (A. F. Huxley and Taylor, 1958).

An alternative explanation is that the responsive element in the present experiments is the SR, and that longitudinal connections exist between adjacent lateral cisternae in the region of the triad. Peachey (1965) has presented a few examples of direct anatomical continuity between the SR of adjacent sarcomeres, but no information is available as to the frequency of such connections.

A third possibility is that the triadic junction in the skinned fiber offers a low resistance to the passage of inorganic ions, so that the entire internal membrane system is connected by the relatively low resistance of the luminal fluid. Although an appreciable conductance between the SR and T system does not appear to be present in the intact fiber (Falk and Fatt, 1964), Peachey (1965) has stressed the anatomical similarity of these junctions to the low resistance junctions between epithelial cells (Wiener, Spiro, and Loewenstein, 1964), and the possibility must be considered that, under appropriate conditions, the conductance of these junctions might increase appreciably.
CURRENT STRENGTH  Further evidence that an electrically responsive element of the internal membrane system is continuous longitudinally over many sarcomeres can be obtained from consideration of the current strength required to produce activation. If a single element of the T system or the sarcoplasmic reticulum is approximated by a right circular cylinder 500 Å in diameter, and the membrane constants of this element are assumed to be similar to those of the surface membrane of muscle, the displacement of membrane potential in a longitudinal field will be approximately proportional to the length of the element (see Appendix). For an element extending over just one sarcomere, or about 3 μ, the maximum membrane potential change in an applied field of 10 v/cm, the usual threshold for stimulation in the skinned fiber, would be only about 1.5 mv, a value which seems unlikely to have a significant physiological effect. Continuity of the internal membrane system over about 10 sarcomeres, however, would result in a maximum potential change of about 15 mv.

From similar considerations, it can be shown that the transversely oriented elements of the T system would be expected to develop potential changes of a fraction of a millivolt with the present currents (Sten-Knudsen, 1960) and a direct effect of applied current on the transverse elements of the T system seems unlikely in the present study. If the Z disc were to exclude ionic currents, however, appreciable current density could develop in the region of the T tubule, and much larger potential gradients would result. In this connection, it is of interest that similar responses were readily obtained with similar current strengths (~ 70 ma/cm²) in skinned fiber preparations from the rat gastrocnemius (unpublished experiments) in which the T tubule is located at the level of the A-I junction (Pellegrino and Franzini, 1963).

RELATION OF THE ELECTRICAL RESPONSE OF SKINNED FIBERS TO CALCIUM RELEASE FROM SARCOPLASMIC RETICULUM FRAGMENTS  Lee et al. (1966) have reported that calcium is released from fragments of sarcoplasmic reticulum bathed in a 100 mM KCl medium when electric current is applied; this result differs from that of the present experiments in which a high chloride concentration abolished the response to electrical stimulation, presumably by depolarizing the internal membranes. However, Lee's results were obtained with 1 v stimuli applied to electrodes separated by 3 mm, that is, the potential gradient in the medium across a single SR fragment (maximum diameter ~200 μμ, Ebashi and Lipmann, 1962) was presumably less than 70 μv; this suggests that some effect other than a displacement of membrane potential across a single SR fragment was responsible for calcium release.

SPREAD OF RESPONSE  In the present experiments, the internal membrane system before activation is assumed to be polarized with its lumen positive with respect to the myofibrillar space; a maximum depolarization would
therefore be expected nearest the anode and an increase in current strength above threshold should produce an extension of contraction toward the cathode. When this expectation was tested directly, however, it was found that the spread of contraction actually occurred in both longitudinal directions from the originally responsive sarcomeres (Fig. 2), a result which implies that the threshold membrane depolarization for a given applied field occurs somewhere along the length of the responsive structure rather than at either end. Sten-Knudsen (1960) has pointed out that a variation in electrical constants occasioned by irregularities in the diameter of the responsive element can produce local maxima in membrane polarization. Since the complex geometry of the SR might be expected to give rise to such a variation in the effective electrical constants from one sarcomere to the next, the mode of spread of the graded response to electrical stimulation would seem to implicate the direct participation of potential changes across the SR membranes in the activation process in these experiments.

Evidence for Active Depolarization within the Internal Membrane System

Previous studies on intact muscle have indicated that, although the surface membrane is capable of all-or-none behavior, propagation within the internal membrane system probably occurs by passive spread of a local surface depolarization (A. F. Huxley and Taylor, 1958). In general, the present experiments are in accord with these conclusions. The electrical response, for example, was usually continuously gradable with increasing current strength. Similarly the strength-duration curve for electrical stimulation revealed a chronaxie of about 10 msec, a result suggestive of the passive release of an activator substance rather than the all-or-none responsiveness of an excitable membrane.

On rare occasions, however, the first electrical stimulus applied to a skinned fiber triggered an extensive response which propagated for up to 200 μ within the skinned region while subsequent stimulation of the same preparation then gave the more usually encountered gradable response. Natori (1965) has also reported that in certain cases the application of current to skinned fibers from the Japanese toad could elicit a propagating contraction that appeared to begin at the anode and move as a "bead" towards the cathode. Such observations suggest that the internal membrane system is capable, under certain unknown conditions, of a regenerative response, and this conclusion is strengthened by the finding of spontaneous periodic contractions in the skinned fiber (Fig. 5).

While it is generally accepted that the final step in activation is the release of calcium into the myofilament space, one difficulty with this hypothesis has been that the passive currents which would be expected to flow across the internal membranes during the action potential are inadequate to provide one charged activator ion per molecule of contractile protein. As Freygang
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Depolarization of Internal Membranes  

(1965) has pointed out, an electrically regenerative process within the internal membrane system would remove this difficulty, since a small passive displacement of the potential across the internal membranes, by altering the permeability of the internal membrane system to some cation concentrated within its lumen, would result in a flux of this cation across the membrane and would further displace the membrane potential. Furthermore, if such a process were to involve the permeability of the SR membranes to calcium, it could, by discharging calcium into the myofilament space, serve as the final step in excitation-contraction coupling.

APPENDIX

Membrane Polarization of a Cylinder in a Homogeneous Longitudinal dc Field
(Modified from Sten-Knudsen, 1960)

Let
\[ e = \text{undisturbed parallel dc field (v/cm)} \]
\[ x = \text{distance along axis from midpoint of cylinder} \]
\[ 2L = \text{length of cylinder (cm)} \]
\[ a = \text{radius of cylinder (cm)} \]
\[ V_m = \text{potential difference across cylinder wall in excess of normal resting potential} \]
\[ I = \text{longitudinal current in cylinder} \]
\[ \lambda = \text{dc space constant of cylinder} \]

Assuming that the disturbing effect of the cylinder on the external field is small, the equation to be solved for the steady-state value of \( V_m \) is:

\[ \frac{d^2 V_m}{dx^2} = \frac{V_m}{\lambda^2} \]

with the conditions (1) \( V_m(0) = 0 \), (2) \( I(L) = I(-L) = 0 \), or \( \frac{dV_m}{dx} = -e \) for \( x = \pm L \).

The solution is:

\[ V_m = -e \lambda \left( \frac{\sinh x/\lambda}{\cosh L/\lambda} \right) \]

Taking \( \rho_s = 3100 \, \Omega \, \text{cm}^2 \), the resistivity of the surface membrane of muscle (Falk and Fatt, 1964), \( \rho_i = 80 \, \Omega \cdot \text{cm} \), the resistivity of Ringer's fluid, and \( a = 250 \, \mu \text{m} \), we obtain \( \lambda \approx 70 \, \mu \text{m} \).

For a cylinder one sarcomere long (3\( \mu \text{m} \)), \( L = 1.5 \mu \text{m} \). Then \( \lambda \gg L \), \( \cosh L/\lambda \approx 1 \), \( \sinh x/\lambda \approx x/\lambda \) and

\[ V_m \approx -ex \]

The usual threshold current for stimulation of the skinned fiber preparation was about 4 \( \mu \text{A} \) in a fiber 100 \( \mu \text{m} \) in diameter. Assuming uniform current distribution through the fiber cross-section, the current density would be 50 \( \text{mA/cm}^2 \). Taking 200 \( \Omega \cdot \text{cm} \) as the specific resistivity of the sarcolemma (Falk and Fatt, 1964), \( e = 10 \, \text{v/cm} \) and \( V_m(L = 1.5 \u{03bc}m) \approx 1.5 \text{mv} \).

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