Contractile Activation
in Frog Skeletal Muscle

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ABSTRACT  Contractile activation was studied in frog single muscle fibers treated with tetrodotoxin to block action potentials. The membrane potential in a short segment of the fiber was controlled with a two-electrode voltage clamp, and the contractile response of superficial myofibrils in this segment was observed microscopically. The strength-duration relation for contractile activation was similar to that reported by Adrian, Chandler, and Hodgkin (1969); at 3.9°C, the contraction threshold was -44 mV for long depolarizing pulses (100-ms) and increased to +64 mV for 2-ms depolarizations. Hyperpolarizing postpulses shifted the threshold for 2-ms pulses to more positive values, and a similar, but smaller, effect was produced by hyperpolarizing prepulses. The decay of excitability following subthreshold pulses showed two apparently distinct components; at 3.9°C, excitability fell to 50% of its initial value within 4 ms, while the subsequent decline of excitability proceeded with a half-time of about 20 ms.

Adrian, Chandler, and Hodgkin (1969) have examined the relation between depolarization and contractile activation in frog twitch fibers under conditions where the magnitude and the duration of a locally applied depolarization could be controlled by a voltage-clamp technique. Contractions of individual fibers within the sartorius muscle were observed with a relatively low power microscope and, as Adrian, Chandler, and Hodgkin (1969) have pointed out, an “obvious weakness” of this method was that it was not possible to determine the extent of the fiber which was activated with a depolarizing pulse. In the present study, similar experiments were performed on isolated single muscle fibers, so that higher resolution microscopy could be employed to evaluate the contractile response of only a few myofibrils. The results obtained are in general agreement with those of Adrian, Chandler, and Hodgkin (1969), and the increased sensitivity of the present method afforded an opportunity to characterize the time-course of mechanical excitability following a subthreshold pulse.
METHODS

Fiber Preparation

Single fibers dissected from the semitendinosus muscle of Rana pipiens were studied by a two-electrode voltage clamp method (Adrian, Costantin, and Peachey, 1969). A section of the fiber was immobilized by resting it on two Vaseline-coated Lucite pedestals separated by 1.5 mm, and the region of the fiber chosen for study was located between these two pedestals. Fibers were bathed in a normal Ringer solution containing 115 mM NaCl, 2.5 mM KCl, 1.8 mM CaCl₂, and 1.5 mM sodium phosphate at pH 7.1. About 0.5 μg/ml tetrodotoxin was added to prevent action potentials.

Temperature Control

The bath temperature was controlled by perfusion of a coolant solution through glass tubes built into the experimental chamber and through silicone tubing which jacketed the microscope objective. Condensation of water vapor on the bottom surface of the chamber was prevented by directing a stream of dry air against the surface. The temperature was routinely determined at the end of each experiment by displacing the region of the fiber under study with a temperature probe about 800 μm in diameter. With a given setting of the control system, the temperature varied by less than ±0.5°C in different experiments.

Electrical Stimulation

An isolated single fiber was impaled by two microelectrodes; one served to record the intracellular potential and the second, inserted diametrically opposite the first, served to pass current from an appropriate feedback circuit. The feedback circuit had an open loop gain of $1.4 \times 10^5$ and a maximum output of ±80 V. A third microelectrode was positioned extracellularly about 50–70 μm away from the intracellular recording electrode, and the membrane potential was taken as the potential difference between these two electrodes. Fibers were clamped at a holding potential of −90 mV. Total clamping current was recorded with a virtual ground circuit with a time constant of 1 ms. The current record served primarily to monitor membrane damage produced by the microelectrode impalements.

The recorded waveform of the membrane potential in response to rectangular command pulses was dependent on the characteristics of the microelectrodes and of the muscle fiber and on the adjustment of the feedback circuit. The recorded depolarizing pulse usually rose to 90% of its final value within 0.25 ms. Pulse duration was measured at half-amplitude from the holding potential in all experiments.

Visual Observations

The optical apparatus has been described previously (Costantin and Taylor, 1973). The depth of field of the microscope image was less than 10 μm so that an optical section of the fiber could be obtained. Fiber thickness was measured as the range of movement of the microscope in focusing from the top to the bottom of the fiber. The plane of focus was then set at the midpoint of this range, and the fiber was photographed for
subsequent measurement of fiber width and striation spacing. Both intracellular microelectrodes were inserted in this focal plane.

**DETERMINATION OF THE MECHANICAL THRESHOLD** Since potential gradients can exist within the T system following surface membrane depolarization, (Huxley and Taylor, 1958; Gonzalez-Serratos, 1966, 1971; Adrian, Costantin, and Peachey, 1969; Costantin, 1970), it would be desirable, in studying the voltage dependence of contractile activation, to examine the mechanical response of myofibrils within a region of the muscle fiber where the T-tubule membrane potential is known. Although direct recording from T-tubule elements is not possible, the difference between the recorded surface membrane potential and the T-tubule membrane potential should be smallest in the tubular elements closest to the fiber surface; accordingly, the contractile response to voltage-clamp depolarizations was monitored in the myofibrils within this region. This was accomplished by readjustment of the plane of focus of the microscope, after microelectrode impalement, so that the striations on the upper (or lower) surface of the fiber were just visible, and the mechanical response of the myofibrils in this region was observed.

For each threshold determination, the pulse duration was set first, and pulses of varying amplitude were applied to the fiber. Both the amplitude of the pulse and the time of application were controlled by the observer. On a few occasions, however, the pulse duration was inadvertently not altered between two successive threshold determinations, so that the observer was not aware that the same pulse duration was being repeated. The thresholds determined on the two runs were quite similar.

It was usually possible to apply pulses every 3–5 s with no evidence of interaction between the individual pulses, and a single threshold determination required 30 s or less. In some fibers maintained at 3–4°C, however, individual pulses clearly interacted if the interval between pulses was much less than 10 s. Usually this interaction was evident as a progressively weaker response to successive pulses of identical size. Rarely however, the opposite effect was seen: Repetition of a subthreshold pulse at 1-s intervals resulted in a contraction while a single application of an identical pulse did not. These phenomena were not examined in any detail, but they appeared to be more pronounced with brief pulses, e.g. 2–3-ms duration. If the interval between test pulses was greater than about 10 s, reproducible determinations of the mechanical threshold could be obtained in these fibers. However, this long interval between pulses did limit the amount of data which could be obtained on an individual fiber, especially since fibers kept at low temperatures seemed more likely to develop permanent contractures at the sites of electrode impalement.

**ACCURACY OF THE MEASUREMENTS** With pulse durations longer than 10 ms, a readily detectable decrease in striation spacing usually appeared with an increment in the pulse amplitude of about 1 mV. With briefer pulses, however, the contraction threshold was more difficult to determine, and this was particularly the case in fibers studied at 3–4°C, where the slow speed of shortening made it difficult to recognize a weak mechanical response. With pulses of 2–3-ms duration, the transition from no contraction to an unequivocal contraction usually required a 4–6-mV increase in the amplitude of the depolarizing pulse. The threshold was taken as the midpoint of this
range. With still briefer pulses in the range where the strength-duration relation rose steeply, the difference between pulses which elicited either no response or an unequivocal contraction was as large as 10–20 mV, and such brief pulses were not routinely employed.

The mechanical threshold for the longer pulses was quite reproducible. In 35 of the fibers studied, the threshold for 100-ms pulses was determined at the beginning and at the end of the experiment, and the difference between the two determinations was $0.4 \pm 0.3$ mV (Mean $\pm$ SEM). The threshold for brief pulses was more variable, and in some fibers, progressively larger pulses were clearly required to elicit a just-detectable mechanical response. There was no apparent reason for this shift in threshold. In the fiber illustrated in Fig. 3, for example, the prolonged impalement with two microelectrodes and the production of repeated local contractions apparently produced little membrane damage, since the membrane potential, which was initially $-85$ mV, was found to be $-82$ mV at the end of the experiment. Nevertheless, the threshold for 2-ms pulses increased progressively from $+44$ to $+50$ mV in the course of the experiment. In some other fibers in which the experiment was terminated because large contractures developed at the microelectrode site (usually at the current-passing electrode), little or no shift in the contraction threshold was observed.

**Nonuniformities of Membrane Potential**

The first sign of contraction when depolarizing steps of increasing amplitude are applied to a voltage-clamped muscle fiber is a small local contraction at the site of impalement with the current-passing electrode (Adrian, Costantin, and Peachey, 1969). In the present study, because of the shallow depth of field of the optical system, this local response was usually not visible when the microscope was focused on the upper (or lower) surface of the fiber. On occasion, the local response at the site of electrode impalement did produce some lateral movement of the superficial myofibrils under observation, but this movement was readily distinguished from the shortening of the striation spacing seen at the contraction threshold.

This local response at the current electrode arises because the small current source gives rise to a local electric field within the sarcoplasm so that depolarization is not uniform about the circumference of the fiber. If the fiber is approximated as a right circular cylinder, a quantitative description of the circumferential variation of surface membrane potential is available for steady-state depolarizations (Adrian, Costantin, and Peachey, 1969; Eisenberg and Johnson, 1970); the membrane potential at the recording electrode and at the upper (or lower) surface of the fiber should differ by less than 2%. A similar description of the circumferential variation of potential during a transient depolarizing step has thus far proven to be an intractable problem, but an estimate of the variation can be obtained by considering the response to the application of an appropriate sinusoidal voltage. The maximum rate of rise of just-threshold 2-ms depolarizing pulses was slightly less than 700 V/s, roughly equivalent to a sine wave of 1,400 cps. If the effective capacitance of the muscle fiber at this frequency is taken as 2 $\mu$F/cm$^2$, the effective length constant for a fiber 80 $\mu$m in diameter is about 400 $\mu$m, and the predicted difference between the recorded membrane potential and the potential at the upper surface of the fiber is 3% (See Table 2 of Eisen-
berg and Johnson, 1970). Although the rise time of the applied depolarization will be shorter at the upper surface of the fiber than at the recording site, the recorded rise time is a small fraction of the pulse duration, even for the briefest pulses employed in this study; thus the discrepancy between the pulse durations at the two sites cannot be very large. These considerations suggest that the recorded wave form of the depolarizing pulse is quite similar to the surface depolarization at the site where the mechanical response is monitored. This conclusion is supported by the experimental observation that, aside from the immediate neighborhood of the microelectrodes, no difference in contraction threshold of superficial myofibrils was detectable when the plane of focus was altered to examine the entire fiber circumference.

RESULTS

Strength-Duration Relation

Adrian, Chandler, and Hodgkin (1969) have shown that in voltage-clamped frog muscle fibers, the amplitude of the depolarizing pulse required to elicit a just-detectable mechanical response increased as the pulse duration decreased. For depolarizations to membrane potentials more positive than \(-10\) mV, this strength-duration relation could be fitted by a simple hyperbolic curve:

\[(V + C)t_c = B_T\] (1)

where \(C\) and \(B_T\) are constants, \(V\) is the membrane potential, and \(t_c\) the pulse duration for a just-threshold depolarizing step. Similar results were obtained in the present study. Fig. 1 illustrates the strength-duration relations of two single fibers obtained from the same frog which were studied at 2.8°C. The curves fitted to the data for brief pulses were calculated from Eq. 1; the curve-fitting method is described in the figure legend. Adrian, Chandler, and Hodgkin (1969) found that \(B_T\) was roughly similar in different fibers and equal to about 120 mV · ms at 4°C. This was not the case in the present experiments. In the fibers in Fig. 1, for example, the values of \(B_T\) differ by almost two-fold. Because of this large fiber-to-fiber variation in the strength-duration relation, the contraction thresholds for a range of pulse durations were routinely determined as a preliminary to further experimentation in each fiber examined.

Variation in the strength-duration relation was particularly striking at the lowest temperatures employed in the present study, about 3–4°C, and was mainly due to the large variation in contraction threshold with brief pulses; for fibers studied at 3.9°C, the standard deviation of the threshold for 100-ms pulses was 2.8 mV while, for 2-ms pulses, the standard deviation was 25.5 mV (see Fig. 2). No explanation for this variation is apparent. Most fibers were stretched to striation spacings of about 3 μm before study, since this facilitated their impalement with microelectrodes. A few fibers with striation
Figure 1. The strength-duration relation for contractile activation. The results obtained in two different single fibers from the same frog are plotted. In the fiber represented by the open circles, the contraction threshold for a 100-ms pulse was determined first, and the pulse duration was progressively decreased to 2 ms. In the fiber represented by the filled circles, the contraction threshold for a 100-ms pulse was determined first, and the pulse duration was progressively decreased to 3 ms and then increased to 500 ms. Finally the thresholds for 20- and 100-ms pulses were redetermined. Temperature: 2.8°C. The curves were drawn from Eq. 1; the membrane potential for the data points more positive than $-10\,\text{mV}$ were plotted against the reciprocal of the pulse duration, and a straight line was fitted by eye to determine $C$ and $B_T$. For the upper curve: $C = 40\,\text{mV}$, $B_T = 336\,\text{mV}\cdot\text{msec}$. For the lower curve: $C = 35\,\text{mV}$, $B_T = 182\,\text{mV}\cdot\text{ms}$.

Figure 2. Effect of temperature on the strength-duration relation. Open circles: mean of 11 fibers at 3.9°C. Filled circles: mean of 18 fibers at 8.7°C. Squares: mean of 4 fibers at 22.1°C. The curves were drawn from Eq. 1; the values of $C$ and $B_T$ which were employed are given in Table I. The brackets are $1\,\text{SD}$ of the measurements at each pulse duration.
spacings below 2.5 μm were also examined, however, and the contraction thresholds of these fibers were not obviously different from those of the stretched fibers.

**EFFECT OF TEMPERATURE** The effect of temperature on the strength-duration relation is illustrated in Fig. 2, in which the mean values of the contraction threshold over a range of pulse durations have been plotted for three different temperatures, 3.9°, 8.7°, and 22.1°C. There is some shift in the threshold for 100-ms pulses toward more depolarized levels at the lower temperatures, but a much larger effect is seen on the threshold for brief depolarizations. In fibers studied at 3.9° and at 8.7°C, Eq. 1 provided a good fit to the data; the temperature dependence of the contraction threshold for brief pulses is reflected in an increase in the mean value of $B_T$ from 114 mV · ms at 8.7°C to 192 mV · ms at 3.9°C. It should be noted that the decreased velocity of shortening at 3.9°C made visual detection of just-threshold contractions with 2-3-ms pulses quite difficult (see Methods), and this may in part account for the striking temperature dependence observed.

The mean values of the contraction thresholds and of the parameters $B_T$ and $C$ are presented in Table I. Some early studies were carried out on a series of fibers at 3.0°C, and these results have been listed separately in Table I.

**RHEOBASE** The contraction threshold for 100-ms pulses is close to the rheobase value. Longer pulses were tested in four fibers, two at 3°C and two at 8.7°C; with pulses 200-1,000 ms in duration, the threshold amplitude was 1-3 mV less than for a 100-ms pulse.

**Effect of Postpulses on the Strength-Duration Relation**
In this series of experiments, a depolarizing pulse was followed immediately by a hyperpolarizing or depolarizing postpulse 100 ms in duration, and the effect of the postpulse on the amplitude of the depolarizing pulse required to elicit a just-detectable contraction was determined. In the fiber illustrated in Fig. 3, it was possible to carry out five separate determinations of the strength-duration relation with pulses of 2-100 ms in duration. The first, third, and fifth runs were with a depolarizing pulse alone; in the second run (filled circles), the depolarizing pulse was followed by a 30-mV hyperpolarizing postpulse and in the fourth run (open circles), by a 30-mV depolarizing postpulse. The threshold for 2-ms pulses shifted from an initial value of +44 to +47.5 mV on the third run and to +50 mV on the fifth run, while the 10- and 100-ms thresholds were not detectably different. As can be seen in Fig. 3, the amplitude of a brief pulse required to elicit a threshold mechanical response was less when the pulse was followed by a depolarization and greater


<table>
<thead>
<tr>
<th>Temperature°C</th>
<th>Number of fibers</th>
<th>Membrane potential V</th>
<th>Fiber diameter μm</th>
<th>Striation spacing</th>
<th>Pulse duration ms</th>
<th>Bf</th>
<th>C</th>
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<tr>
<td>3.0</td>
<td>13</td>
<td>-84</td>
<td>77</td>
<td>2.83</td>
<td>-46.2</td>
<td>19.3</td>
<td>+5.8</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(±1.1)</td>
<td>(±2.4)</td>
<td>(±4.2)</td>
</tr>
<tr>
<td>3.9</td>
<td>11</td>
<td>-83</td>
<td>76</td>
<td>3.30</td>
<td>-44.2</td>
<td>18.3</td>
<td>+3.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(±0.8)</td>
<td>(±1.4)</td>
<td>(±2.4)</td>
</tr>
<tr>
<td>8.7</td>
<td>18</td>
<td>-86</td>
<td>82</td>
<td>3.12</td>
<td>-44.8</td>
<td>-29.1</td>
<td>-15.7</td>
</tr>
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<td></td>
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<td></td>
<td></td>
<td>(±0.8)</td>
<td>(±1.1)</td>
<td>(±1.4)</td>
</tr>
<tr>
<td>8.7**</td>
<td>2</td>
<td>-94</td>
<td>72</td>
<td>3.30</td>
<td>-45.2</td>
<td>-30.2</td>
<td>-18.0</td>
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<td>(±0.3)</td>
<td>(±1.3)</td>
<td>(±2.5)</td>
</tr>
<tr>
<td>22.1</td>
<td>4</td>
<td>-86</td>
<td>78</td>
<td>2.94</td>
<td>-51.4</td>
<td>-46.2</td>
<td>-40.9</td>
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<td></td>
<td></td>
<td>(±2.0)</td>
<td>(±1.5)</td>
<td>(±2.2)</td>
</tr>
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</table>

The numbers in parentheses are 1 SEM.

* Potential recorded following insertion of both microelectrodes.

† Calculated as $(\text{width} \times \text{depth})^{1/2}$.

§ Each column gives the contraction threshold in millivolts for the pulse duration shown.

∥ Fibers bathed in 0.18 mM Ca++; 3.6 mM Mg++ Ringer.
when followed by a hyperpolarization; no effect of the postpulse was observed with 100-ms test pulses.

Because of the large fiber-to-fiber variation in the contraction threshold for brief pulses, it was not practical to compare the effect of postpulses (or other experimental variables) in different fibers in terms of membrane potential, and therefore the formalism of Eq. 1 was adopted to express the experimental data. As Adrian, Chandler, and Hodgkin (1969) have pointed out, the quantity $B_mV \cdot ms$ in Eq. 1 can be taken to represent the effective area of a just-threshold brief pulse, that is, the area of the portion of the voltage-time record beyond a membrane potential of $-C \text{ mV}$. In the present studies, the value of $C$ for each fiber was determined from the strength-duration relation, and the effective area, $B$, of a test pulse was measured on a tracing made from the oscilloscope record of the pulse. The threshold for a control pulse of the same duration as the test pulse was routinely determined before and after each series of test pulses, and the values of $B_T$ were averaged. The relative magnitude of the test pulse was then expressed as $B/B_T$. In the experiment shown in Fig. 3, for example, $C$ was 42 mV, and $B_T$ for the two control runs which bracketed the test run with hyperpolarizing postpulses was 188 mV \cdot ms for a 2-ms pulse and 190 mV \cdot ms for a 3-ms pulse. In the test run, $B$ measured 218 mV \cdot ms and 213 mV \cdot ms for 2- and 3-ms pulses, respectively. Thus the posthyperpolarization shifted the mechanical threshold to 1.16-1.12 \times the control value.

It should be noted that this method of analysis assumes that the value of

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**Figure 3.** Effect of postpulses on the strength-duration relation. Triangles: mean value of the contraction thresholds determined without postpulses. The brackets about each data point indicate the range of values observed. The curve was drawn from Eq. 1. Filled circles: contraction thresholds determined with a 100-ms postpulse to $-120 \text{ mV}$. Open circles: contraction thresholds determined with a 100-ms postpulse to $-60 \text{ mV}$. Temperature = 2.9°C.
$C$ is not changed by the postpulse. Although both the hyperpolarizing and depolarizing runs in Fig. 3 could be fit by Eq. 1 with an unchanged value of $C$, this point was not tested further, and in most fibers, the effect of postpulses was examined only with 2–3-ms test pulses. This shortened the duration of the experiment and thus avoided large shifts in the contraction threshold for the control pulses which bracketed the test pulse. The effects of hyperpolarizing and depolarizing postpulses on the contraction threshold for 2–3-ms pulses are summarized in Table II.

**EFFECT OF POSTPULSE DURATION** In one fiber studied at 3.9°C, the duration of the postpulse (to $-120$ or $-70$ mV) was varied. In this fiber $B/B_T$ was 1.16 for a 100-ms posthyperpolarization and 0.89 for a 100-ms post-depolarization. A decrease in postpulse duration to 5 ms was without effect on $B/B_T$; when the postpulse duration was shortened to 2 ms however, $B/B_T$ decreased to 1.11 with a posthyperpolarization and increased to 0.96 with a postdepolarization.

**Temporal Summation of Subthreshold Pulses**

**BRIEF PULSES** Adrian, Chandler, and Hodgkin (1969) have reported that the effect of a brief subthreshold pulse on the activation process decayed by 50% within 2–6 ms, but because of the uncertainty of the visual end point in their experimental method, the time-course of the decay of mechanical excitability could not be accurately determined. With the more sensitive method of detecting a threshold mechanical response employed in the present study, however, it was possible to explore the decay of excitability in more detail. The experimental protocol was similar to that employed by Adrian,

<table>
<thead>
<tr>
<th>Membrane potential during postpulse</th>
<th>Temperature</th>
<th>Number of fibers</th>
<th>$B/B_T^*$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$mV$</td>
<td>°C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$-60$</td>
<td>3.0</td>
<td>3</td>
<td>0.73 ($\pm0.037$)</td>
</tr>
<tr>
<td></td>
<td>8.7</td>
<td>3</td>
<td>0.83 ($\pm0.023$)</td>
</tr>
<tr>
<td>$-70$</td>
<td>3.9</td>
<td>1</td>
<td>0.89</td>
</tr>
<tr>
<td>$-75$</td>
<td>3.0</td>
<td>1</td>
<td>0.90</td>
</tr>
<tr>
<td>$-120$</td>
<td>3.0, 3.9</td>
<td>3</td>
<td>1.16 ($\pm0.009$)</td>
</tr>
<tr>
<td></td>
<td>8.7</td>
<td>2</td>
<td>1.12 ($\pm0.042$)</td>
</tr>
<tr>
<td>$-150$</td>
<td>3.0</td>
<td>2</td>
<td>1.31 ($\pm0.043$)</td>
</tr>
<tr>
<td></td>
<td>8.7</td>
<td>3</td>
<td>1.19 ($\pm0.017$)</td>
</tr>
</tbody>
</table>

The test pulse was 2–3 ms in duration, and the postpulse was 100 ms in duration.

$^*$ $B/B_T = \text{effective area of test pulse (with postpulse)} / \text{effective area of control pulse}$. The numbers in parentheses are $\pm$ SEM.
Chandler, and Hodgkin (1969). Two brief (2- or 3-ms) depolarizing pulses separated by various time intervals were applied to the fiber; the amplitude of the first pulse \( (V_1) \) was set so that its effective area \( (B_1) \), the shaded portion of \( V_1 \) in the inset of Fig. 4) was 80–90% of the threshold value, \( B_T \), for the fiber, and the amplitude of the second pulse \( (V_2) \) was increased until \( V_1 + V_2 \) elicited a just-detectable contraction. The effective area of \( V_1 \) \( (B_2) \), the shaded portion of \( V_2 \) in the inset of Fig. 4) was measured, and \( B_T - B_2 \) was taken as a measure of the excitability induced by \( V_1 \) which was still present after the delay interval. The results obtained on a series of fibers at 3.9 and 8.7°C are plotted in Fig. 4. The data have been normalized to the initial area of \( V_1 \), i.e., the data are plotted as \( (B_T - B_2)/B_1 \) for each delay interval. In agreement with the results of Adrian, Chandler, and Hodgkin (1969), the excitability produced by a brief subthreshold pulse decayed by about 50% in 2–4 ms. However, a second slower component of the decay of excitability was also

![Figure 4](image_url)  
**Figure 4.** Decay of excitability following near-threshold brief pulses. The depolarizing pulses which were applied to the fiber are shown diagramatically in the inset; the area of each pulse beyond a membrane potential of \(-C\) mV is indicated by shading. The effective area of \( V_1 \), calculated as described in the text, is plotted on a logarithmic scale against the interpulse interval. Open circles: mean (± SEM) of three fibers at 3.9°C. The interrupted line has a half-time of 20 ms. Filled circles: mean (± SEM) of three fibers at 8.7°C.

![Figure 5](image_url)  
**Figure 5.** Effect of membrane potential on decay of excitability. The pulses which were applied to the fiber are shown diagramatically in the inset. The effective area of \( V_1 \), calculated as described in the text, is plotted on a logarithmic scale against the interpulse interval. All data points are from the same fiber studied at 3.9°C. Circles: membrane potential during interpulse interval = \(-90\) mV. Squares: membrane potential during interpulse interval = \(-120\) mV. Open symbols: data points obtained with progressive increases in \( \Delta t \). Filled symbols: repeat determination with \( \Delta t \) decreased to 2 ms.
present; in Fig. 4, the straight line drawn through the data points from 11 to 24 ms at 3.9°C has a half-decay time of 20 ms.

**EFFECT OF MEMBRANE POTENTIAL DURING DELAY INTERVAL** The observation that a posthyperpolarization decreases the effectiveness of a brief pulse in eliciting contractile activation (see Table II) suggests that the activation process continues to increase for a few milliseconds after the end of a brief depolarization and that the time-course of deactivation of this process is dependent on the membrane potential immediately after the pulse. Accordingly, it might be anticipated that the decline of excitability after a subthreshold pulse would also be sensitive to the membrane potential, and, in fact, a more rapid decay of excitability was found when the holding potential during the interval between two brief subthreshold depolarizations was set at a hyperpolarized level. A typical experiment is illustrated in Fig. 5; both the rapid and the slow component of decay appeared to be accelerated by a 30-mV hyperpolarization applied during the interval between V₁ and V₂.

**EFFECT OF PREPULSE ALONE** Surprisingly, if V₁ was omitted in the pulse sequence shown in Fig. 5, the amplitude of V₂ required to elicit a threshold contraction was increased by a few millivolts when V₂ was preceded by a hyperpolarizing prepulse. Although the shifts in threshold were consistently rather small, the effect of a hyperpolarizing prepulse was quite readily demonstrated. Thus if the amplitude of a brief pulse was adjusted to elicit an unequivocal contraction of superficial myofibrils, application of the same pulse preceded by a 30-mV hyperpolarization gave no mechanical response, while omission of the prehyperpolarization again resulted in a contraction.

This effect was investigated in a series of fibers by applying 2–3-ms test pulses with and without prepulses of various amplitudes. The results are summarized in Table III; the data are recorded as B/B₀, the effective area of a just-threshold test pulse with a prepulse divided by the effective area of a just-threshold control pulse.

The finding that a hyperpolarizing prepulse shifts the contraction threshold casts doubt on the conclusion that the rate of the slow component of decay of excitability illustrated in Fig. 5 is increased by hyperpolarization. The combination of V₁ + V₂ separated by a time interval during which the membrane potential is held at −120 mV can be considered as an initial pulse V₁ followed by a hyperpolarizing postpulse and a second pulse V₂ preceded by a hyperpolarizing prepulse. If the prepulse is assumed to decrease the effectiveness of V₂ by about 6% (see Table III) and the data points in Fig. 5 for the hyperpolarizing interval are corrected for this, the slow component of the decay of V₁ is quite similar to that seen without hyperpolarization.

**LONG PULSES** Adrian, Chandler, and Hodgkin (1969) reported that a long pulse which was just below rheobase produced a relatively small increase
**TABLE III**

**EFFECT OF PREPULSES ON CONTRACTILE ACTIVATION**

<table>
<thead>
<tr>
<th>Membrane potential during prepulse</th>
<th>Prepulse duration</th>
<th>Temperature °C</th>
<th>Number of fibers</th>
<th>B/B Tr*</th>
</tr>
</thead>
<tbody>
<tr>
<td>-70 mV</td>
<td>5 ms</td>
<td>3.9</td>
<td>3</td>
<td>0.97 (±0.015)</td>
</tr>
<tr>
<td></td>
<td>4 ms</td>
<td>8.7</td>
<td>1</td>
<td>0.98</td>
</tr>
<tr>
<td>-120 mV</td>
<td>5 ms</td>
<td>3.9</td>
<td>3</td>
<td>1.06 (±0.012)</td>
</tr>
<tr>
<td></td>
<td>4-8 ms</td>
<td>8.7</td>
<td>4</td>
<td>1.05 (±0.016)</td>
</tr>
<tr>
<td>-150 mV</td>
<td>5 ms</td>
<td>3.9</td>
<td>3</td>
<td>1.10 (±0.032)</td>
</tr>
</tbody>
</table>

The test pulse was 2-3 ms in duration.

* B/B Tr = effective area of test pulse (with prepulse) divided by effective area of control pulse. The numbers in parentheses are 1 SEM.

in mechanical excitability, since a brief pulse applied immediately afterwards had to be at least 50% of threshold to elicit a visible contraction. Similar results were obtained in the present experiments and are illustrated in Fig. 6. A subthreshold 100-ms depolarizing pulse (V1) was immediately followed by a 2-ms depolarizing pulse, (V2), and the amplitude of V1 was increased until a just-detectable contraction was elicited. As the amplitude of V1 was increased from about 10 mV below threshold to 2 mV below threshold, the effective area of V2 decreased from about 80 to 55% of B Tr.

The time-course of decay of the excitability induced by 100-ms pulses was studied in a series of fibers at 3.9 and at 8.7°C. A brief test pulse (V2 in the inset of Fig. 7) was applied at various times after a subthreshold 100-ms pulse (V1); as discussed previously in connection with Fig. 4, B Tr - B2 was taken as a measure of the excitability induced by V1 which was present at the onset of V2. The results are plotted in Fig. 7; the data have been normalized to the value of B Tr - B2 obtained with zero separation between the two pulses. The amplitude of V1 was set at 2-3 mV below threshold, and the excitability induced by V1, i.e., the initial value of B Tr - B2, ranged from 28-39% of B Tr in the series of fibers at 3.9°C and from 31-50% of B Tr in the fibers at 8.7°C. The decay of excitability showed an initial rapid component and a second slower component which were roughly similar to the two components of decay observed after a brief pulse.

**DECAY OF WEAK SUBTHRESHOLD PULSES** One explanation for the rapid and slow components in the decay of excitability after subthreshold stimuli is that two distinct processes, which decline at different rates, are initiated by depolarization. If this is the case, it might be possible to vary the proportion of each process which is activated by varying the amplitude of the subthreshold pulse. Unfortunately, it was not feasible to study the decay of excitability after larger brief pulses, e.g. 90-95% of threshold, since the
threshold for a single brief pulse could vary by 5–10% in the course of an experiment. On the other hand, the maximum increase in excitability which could be produced by nearly rheobase long pulses was only about 40%, so that if smaller pulses were employed, resolution of the time-course of decay would have been extremely difficult. Thus the one alternative which remained was to investigate the effect of weak brief pulses.

The decay of excitability after a weak brief pulse was compared, in the same fiber, with the decay after a long pulse which produced an apparently equal increment in excitability. As before (see Fig. 7) a 2-ms test pulse was employed to evaluate the mechanical excitability, but the experiment was designed to detect any possible difference between long and brief pulses without relying on the formalism of Eq. 1 for evaluation of the data. A sub-threshold 100-ms pulse ($V_{1}[100]$, represented by the dashed-solid line in the inset of Fig. 8) followed by a 2-ms test pulse ($V_{2}$) was studied first, and the interpulse interval was varied from 0–4 ms. The interval was then reduced to zero, and the amplitude of $V_{2}$ was again determined. With $V_{2}$ held constant, $V_{1}$ was changed to a two-step pulse of 1 ms each ($V_{1}[1 + 1]$, represented by the dotted-solid line in the inset of Fig. 8). The amplitude of the second step was set equal to the amplitude of $V_{1}(100)$, and the amplitude of the first step was varied until $V_{1}(1 + 1) + V_{2}$ produced a just-threshold contraction. With $V_{1}(1 + 1)$ held constant, the interpulse interval was then increased, and $V_{2}$ was varied until a contraction was produced. With this two-step brief pulse,

![Figure 6. Summation of long and short pulses. A 100-ms pulse, $V_{1}$, was followed immediately by a 2-ms pulse, $V_{2}$, and the amplitude of $V_{2}$ was varied until the combination produced a just-threshold contraction. Two fibers are shown, A at 8.6°C and B at 8.5°C. The membrane potential during $V_{1}$ is given on the abscissa, and the threshold for $V_{1}$ alone is indicated by the arrow. The effective area of $V_{2}$, normalized to the effective area of $V_{2}$ alone, is plotted on the ordinate.](image)
FIGURE 7. Decay of excitability after subrheobasic pulses. The depolarizing pulses which were applied to the fiber are shown diagramatically in the inset. The effective area of $V_1$, calculated as described in the text, is plotted logarithmically against the interpulse interval. Open circles: mean (± SEM) of four fibers at 3.9°C. Closed circles: mean (± SEM) of fibers at 8.7°C. The data points for interpulse intervals of 1–4 ms were obtained in seven fibers, and those for interpulse intervals of 8 and 16 ms were obtained in three fibers.

FIGURE 8. Decay of excitability after weak brief pulses. The depolarizing pulses which were applied to the fiber are shown diagramatically in the inset. Two fibers are shown, on the left at 3.9°C and on the right at 3.8°C. The membrane potential during the test pulse, $V_2$, is plotted against the interpulse interval. The threshold amplitude of $V_2$ alone at the beginning and at the end of each experiment is indicated by the interrupted lines (a) and (b), respectively. Circles: data points for the pulse combination $V_1(100) + V_2$. Squares: data points for the pulse combination $V_1(1 + 1) + V_2$. The open circles in the left-hand panel were obtained before and the filled circles after the run with $V_1(1 + 1) + V_2$.

the time-course of $V_2$ was identical after both long and brief pulses with a zero interpulse interval. As the interpulse interval was increased, the amplitude of $V_2$ required to elicit a just-threshold contraction increased much more rapidly after a brief pulse. The results are illustrated in Fig. 8 for two fibers studied at 3.9°C. The circles show the membrane potential of the test pulses which followed a 100-ms pulse and the squares show the potential of test pulses which followed a brief pulse. In both fibers, excitability decays more rapidly after a brief pulse.

When the data of Fig. 8 were analyzed in terms of Eq. 1, the effect of a long pulse declined in 4 ms to an average of 49% of its initial value, while the effect of a brief pulse declined to 15% of its initial value. Thus the slow component of decay of excitability was much less evident after these brief pulses.
Contractile Activation in Low Calcium Solutions

In frog twitch fibers, the increase in calcium permeability and the resultant influx of extracellular calcium during the action potential are quite small (Bianchi and Shanes, 1959), and this calcium influx probably contributes little to the total calcium required for complete activation of the contractile mechanism. In the present experiments, however, just-threshold contractions of myofibrils immediately below the surface membrane were studied, and it seemed possible that a small influx of extracellular calcium could be important under these conditions. Accordingly, contractile activation was investigated in two fibers in a bathing solution in which the calcium concentration had been reduced to 0.18 mM; MgCl₂ (3.6 mM) was added to prevent the shift in contraction threshold which results from a decrease in the divalent cation concentration (Costantin, 1968). This 10-fold decrease in extracellular calcium had no obvious effect on contractile activation; the strength-duration relation of these fibers was identical to that of fibers in normal Ringer at the same temperature (see Table I). In one fiber, the decay of excitability after a brief pulse (2-ms duration; \( B = 0.79 B_T \)) was studied; both rapid and slow components of decay were observed.

DISCUSSION

There is some evidence to suggest that contractile activation, and presumably calcium release, can be graded by varying the amplitude of membrane depolarization beyond the contraction threshold (Hodgkin and Horowicz, 1960; Costantin and Taylor, 1973), and it might be expected that calcium release would also be detectable after subthreshold depolarizations. In the present study, the decay of excitability after subthreshold depolarizations was found to consist of two components; at 3.9°C excitability fell to 50% of its initial value within 4 ms, while the subsequent decline of excitability proceeded with a half-time of about 20 ms (see Fig. 4). Since there is no clear evidence that the decay of the slower component is sensitive to membrane hyperpolarization, it is attractive to speculate that this component reflects the rate of removal of calcium from the myofibrillar space. Thus, a depolarizing pulse could be supposed to initiate some process within the T-system membranes which regulated the release of calcium; after repolarization, the membrane process would rapidly deactivate and the released calcium would then be removed more slowly from the myofibrillar space, presumably by active transport into the sarcoplasmic reticulum. Alternatively, it might be supposed that the released calcium could diffuse into deeper regions of the fiber where attenuation of the applied depolarization had resulted in less calcium release. This possibility seems less likely, however, since the rate of decay of the slow component appears to be rather similar following near-
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threshold long and brief pulses (cf. Figs. 4 and 7), while attenuation along the T system should be more pronounced for brief pulses (Adrian, Costantin, and Peachey, 1969).

Effect of Pre- and Postpulses on Contractile Activation

A shift in the contraction threshold produced by hyperpolarizing or depolarizing postpulses was first reported by Adrian, Chandler, and Hodgkin (1969); they suggested that this effect might have arisen either from a time lag in the transmission of surface membrane potential changes along the T system or from a direct effect of the postpulse membrane potential on the activation process. In the present study in which contractile activity was monitored in superficial myofibrils, any transmission delay along the T system should be very small; nevertheless similar threshold shifts were observed (see Fig. 3). Moreover, the finding that the threshold shifts are greater with postpulses than with prepulses of equal amplitude (cf. Tables II and III) is not consistent with a simple attenuation of the test pulse by the cable properties of the T system. Thus it seems reasonable to conclude that the mechanism of activator release itself is affected by the postpulse membrane potential, presumably because the time-course of deactivation of the release process is potential dependent. This conclusion is, of course, not particularly surprising; a similar effect is seen in deactivation of the conductance changes which underlie the action potential in excitable cells and leads to the "tail" of sodium or potassium current which follows a brief depolarizing pulse. Schneider and Chandler (1973) have described a voltage-dependent charge movement in skeletal muscle which may be a step in mechanical activation. The rate at which this charge returns to its resting position after repolarization has also been found to be dependent on membrane potential (W. K. Chandler, personal communication).

The shift in contraction threshold with hyperpolarizing prepulses raises the possibility that hyperpolarization might delay the activation process. An alternative explanation is that the effect arises from a transmission delay within the T system. If the Adrian, Chandler, and Hodgkin (1969) model of the T system is accepted, and if it is assumed that a superficial annulus of myofibrils about 5 μm thick is activated during a just-detectable contraction, a 30-mV hyperpolarizing prepulse would decrease the effective area of a brief test pulse by only 1–2%, rather less than the experimentally observed prepulse effect. On the other hand, if an access resistance were present at the fiber surface (Adrian and Peachey, 1973), appreciable attenuation of a test pulse might be produced even in the most superficial T tubules. Unfortunately, the cable properties of the T system have not been sufficiently well characterized to decide this point. If the prepulse effect does reflect a property of the activation process itself, a possible analogy might be the effect of
hyperpolarizing prepulses in delaying the activation of the potassium conductance in the squid axon (Cole and Moore, 1960).

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