Depolarization-Contraction Coupling in Short Frog Muscle Fibers

A Voltage Clamp Study

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ABSTRACT Short muscle fibers (1.5 mm) were dissected from hindlimb muscles of frogs and voltage clamped with two microelectrodes to study phenomena related to depolarization-contraction coupling. Isometric myograms obtained in response to depolarizing pulses of durations between 10 and 500 ms and amplitudes up to 140 mV had the following properties. For suprathreshold pulses of fixed duration (in the range of 20–100 ms), the peak tension achieved, the time to peak tension, and contraction duration increased as the internal potential was made progressively more positive. Peak tension eventually saturates with increasing internal potentials. For pulse durations of ≥ 50 ms, the rate of tension development becomes constant for increasing internal potentials when peak tensions become greater than one-third of the maximum tension possible. Both threshold and maximum steepness of the relation between internal potential and peak tension depend on pulse duration. The relation between the tension-time integral and the stimulus amplitude-duration product was examined. The utility of this relation for excitation-contraction studies is based on the observation that once a depolarizing pulse configuration has elicited maximum tension, further increases in either stimulus duration or amplitude only prolong the contractile response, while the major portion of the relaxation phase after the end of a pulse is exponential, with a time constant that is not significantly affected by either the amplitude or the duration of the pulse. Hence, the area under the tension-response curve provides a measure of the availability to troponin of the calcium released from the sarcoplasmic reticulum in response to membrane depolarization. The results from this work complement those obtained in experiments in which intramembrane charge movements related to contractile activation were studied and those in which intracellular Ca^{++} transients were measured.

INTRODUCTION

Voltage clamp techniques using two or three microelectrodes have been exten-
sively employed to study contractile activation phenomena in skeletal muscle
fibers. Contractile thresholds in the region of impalement are determined by
observing movement with a microscope (Adrian et al., 1969; Costantin and
Taylor, 1973; Costantin, 1974).

The use of short muscle fibers, whose length is similar to or shorter than the
fiber length constant, allows an acceptable, although not perfect, longitudinal
control of the fiber membrane potential. Such a preparation has the added
advantage of permitting the measurement of the tension developed by the fiber
under voltage clamp conditions (Heistracker and Hunt, 1969; Bezanilla et al.,
1971, 1972; Caputo and Fernández de Bolaños, 1979).

In this report we present information about several components of the isomet-
ric myogram obtained in response to different pulse configurations. We also
present evidence indicating that the area under the isometric myograms, i.e., the
tension-time integral, is a more useful measure than peak tension of the activator
release process. This integral increases monotonically with internal potential for
potentials more positive than those at which peak tension is saturated. Experi-
ments carried out with a double-pulse procedure reveal the presence of a process
that reduces the activation effectiveness of the second pulse.

The results from this work complement those obtained in experiments in
which intramembrane charge movements related to contractile activation are
studied (Schneider and Chandler, 1973; Adrian, 1978; Schneider, 1981) and
those in which intracellular Ca^{++} transients are measured (Blinks et al., 1978;
Kovács et al., 1979; Miledi et al., 1981, 1983a, b).

METHODS

Muscle Preparation

Bundles of 2–20 fibers, or occasionally single fibers, were dissected from the lumbricalis
muscle of the fourth toe of the hindlimb of *Rana pipiens* (Dunlap, 1960). The fibers in
these muscles are disposed in a double pennate arrangement so that the length of the
individual fibers is only a fraction of the total muscle length. The fibers used in this study
were generally ~1.5 mm in length. Because of their short length, special care had to be
taken to avoid damage caused by inadvertent stretching during dissection and mounting.

Experimental Setup

Fiber bundles were mounted in a Lucite chamber with a thin glass bottom in thermal
contact with two Peltier thermoelectric elements. The experiments were performed at
temperatures between 9 and 15°C. The temperature was maintained constant within
0.5°C by means of an electronic feedback control system that used a thermistor as a
temperature-sensing element and Peltier devices as cooling units.

When finally mounted, one tendon of the bundle was fixed to the chamber, while the
other tendon was attached by means of a short lever to an RCA 5734 transducer for
tension measurement. In a few experiments the transducer output was fed into an
integrator to obtain the time integral of tension.

Voltage Clamp System

The short length of the fibers made feasible the use of a two-microelectrode clamp system (Weidmann, 1952; Jack et al., 1975). A schematic diagram of the experimental arrangement is given in Fig. 1A. The internal potential was measured with respect to ground with a glass microelectrode filled with 3 M KCl. The potential-measuring microelectrode was connected through a Ag-AgCl electrode to an amplifier (NF 1; Bioelectric Instruments, Hastings-on-Hudson, NY) with provision for variable compensation of input capacitance. The solution in the chamber was connected through an agar bridge filled with normal Ringer's fluid to a pool containing 3 M KCl. This pool was connected by a Ag-AgCl electrode to the summing junction of a current-draining amplifier, which provided a virtual ground. Current was passed into the fiber through a second microelectrode filled with 2 M K-citrate. Electrical contact was made with this microelectrode through a Ag-AgCl electrode connected to the output of a control amplifier (model 170; Analog Devices, Inc., Norwood, MA) for voltage clamping or to a high-voltage stimulator with a 100-MΩ resistor in series for current clamping (see Fig. 1A).

After mounting the fiber bundle in the chamber, the voltage microelectrode was inserted in the center of one of the fibers. To reduce three-dimensional effects, the ideal placement of both electrodes should be at a longitudinal separation of one-half of the fiber radius and an angular separation of 45° (Eisenberg and Johnson, 1970). In practice, the current microelectrode was usually inserted at a distance no longer than one fiber diameter from the voltage microelectrode and a large angular separation was used to decrease cross talk. Current clamp pulses were used to check that both electrodes were in the same fiber. The internal potential of the fiber could be clamped by switching from current clamp to voltage clamp mode. To achieve the desired control of the membrane potential in the experiments described in this study, a steady voltage (the holding potential) and up to three separate pulses could be applied through individual resistors to the summing junction of the control amplifier. In addition, the output of the voltage amplifier was also connected to this summing junction through a resistor. The output of the control amplifier had a dynamic range of ±100 V. This allowed passage of sufficient current through the K-citrate electrode to control the internal potential in most situations. The two capacitors shown in Fig. 1 were variable and were used to stabilize the transient response of the clamp system.

Total membrane current was measured with an operational amplifier wired as a current-to-voltage transducer.

Microelectrodes

The tips of the voltage and current microelectrodes were made flexible following a procedure similar to that described by Freygang et al. (1964). In our procedure, rubber cement thinned with benzene was used to cover a portion of the microelectrode shank. After the cement had dried, it formed a resistant coating. The glass under this coat could be broken with the whole tip immersed in water. The coating of rubber cement acted as a flexible sleeve, maintaining electrical contact between the tip and the rest of the electrode.

Solutions

The composition of the saline used was (in millimoles per liter): 2.5 KCl, 115 NaCl, 1.8 $CaCl_2$, 2.15 Na_2HPO_4, 0.85 NaH_2PO_4. To block the sodium current, either 10^{-6} g/ml of tetrodotoxin was added or Tris-Cl was substituted for NaCl on a mole-for-mole basis. In some experiments, 5 mM $MgCl_2$ was added.
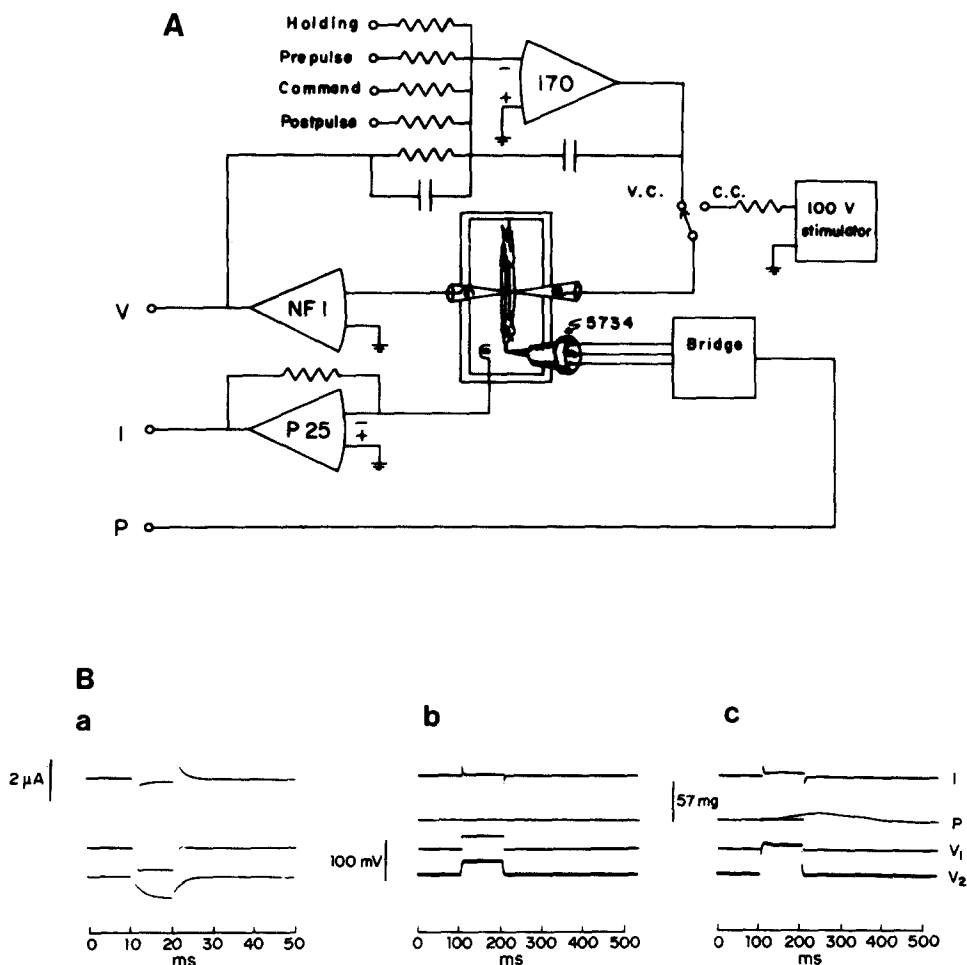
FIGURE 1. (*A*) Schematic diagram of the experimental chamber with a bundle of short muscle fibers, the arrangement of microelectrodes and transducer, and the electronic circuit for voltage clamping and current measurement. (*B*) Tests for longitudinal clamp homogeneity. Potential (V_1 and V_2), current (I), and, in records *b* and *c*, tension (*P*) traces in a voltage clamped short muscle fiber are shown. V_1 represents the membrane potential measured with the control microelectrode inserted in the middle of the fiber and V_2 shows the membrane potential measured at one end of the fiber with a third microelectrode not shown in *A*. The traces in *a* were obtained with a 10-ms hyperpolarizing pulse. Besides showing the relative slowness of the clamp, the records indicate that with short-duration pulses, the potential at the end differs from that achieved at the middle of the fiber, which is in agreement with theoretical analysis (Bezanilla et al., 1982). Records *b* and *c*, obtained with a 100-ms pulse, show that this experimental arrangement is adequate for tension measurement when pulses of relatively long duration are used. The fiber was clamped at a holding potential of −90 mV. Further details are given in the text.

RESULTS

Characteristics of the Lumbricalis Muscle

When electron micrographs of the muscles used in this study were examined, most fibers had the characteristics of twitch muscle fibers. Occasionally, slow fibers are seen (Dr. C. Franzini-Armstrong, unpublished observations).

Measurement of the Passive Electrical Properties of the Short Fibers

The short fiber can be approximated to a simple core conductor cable with the assumption that the tendon terminations are sealed (i.e., no longitudinal current flows). This approximation is valid, provided the frequencies involved are not too high. As our experimental tests of voltage uniformity indicate (see below), the results follow the prediction closely, which indicates that the approximation is valid under our experimental conditions.

In such a preparation, the two-electrode voltage clamp method can be effectively applied for measuring force only when the value of the length constant (λ)

TABLE I

Fiber reference	Total fiber length	Fiber diameter	λ	R_m	R_i
	mm	*μm*	*mm*	*$\Omega \cdot cm^2$*	*$\Omega \cdot cm$*
F1-1	1.71	63	1.41	3,114	246.7
J22-4	1.26	50	1.35	1,230	83.5
J22-1	1.89	63	2.25	1,773	55.1
J22-3	1.35	45	1.50	1,273	60.6
F1-2	1.26	72	1.86	3,553	184.3
F1-3	1.35	63	1.25	1,269	127.9
F5-2	0.99	54	1.45	2,906	186.7
Mean ± SE	1.40±0.11	59±4	1.58±0.13	2,151±381	135.0±27.7

The measurements were carried out at 10–12°C.

is nearly equal to, or larger than, the fiber length (Weidmann, 1952; Caputo and Fernández de Bolaños, 1979). The length constant of several fibers from the lumbricalis muscle was measured using the current clamp approach described in the Methods. Table I gives a summary of the results obtained from seven fibers. It is clear that the value of the length constant is very nearly equal to the total fiber length. The values obtained for R_m and R_i are somewhat lower than those reported for other muscle preparations (Katz, 1948; Falk and Fatt, 1964; Adrian et al., 1970; Hodgkin and Nakajima, 1972). The discrepancy may be due, in part, to the fact that the fibers deteriorated during these measurements, as evidenced by a decline in the resting membrane potential after several impalements. The average resting internal potential for the fibers given in Table I during these measurements was about −50 mV. This contrasts with values of −70 to −80 mV obtained from other fibers used in this study that were only impaled once each by the current and voltage electrodes. For undamaged fibers it is likely that the length constant is longer, in which case the uniformity of the

internal potential along the fiber length would be improved during long voltage clamp pulses.

We obtained an estimate of the DC membrane capacity (C_m) by integrating the ON and OFF capacity transients obtained during voltage clamping. In three fibers, the values of C_m so measured were 7.8, 5.5, and 5.0 $\mu F/cm^2$. These values agree well with those reported for other muscles (Katz, 1948; Falk and Fatt, 1964; Adrian et al., 1970; Hodgkin and Nakajima, 1972). This agreement indicates that the clamping technique used in this study was reasonably effective in controlling the internal potential of the fibers, at least at times longer than the main decay of the capacity transient.

Direct tests for longitudinal uniformity of the internal potential under voltage clamp conditions were made by inserting a third electrode at one end of the fiber. Fig. 1B shows the results obtained with one fiber for different pulse configurations.[1] As predicted by theory (Bezanilla et al., 1982), the internal potential at the end of the fiber responds more slowly than at the center and reaches a smaller value. It is important to note, however, that a steady state is attained within 10 ms in the first record of Fig. 1B. In the third record of Fig. 1B, the pulse amplitude was sufficient to elicit a mechanical response. The results obtained in these trial experiments conform to the expectations derived from a theoretical analysis of the dynamical electrical behavior of a short cable under voltage clamp conditions (Fig. 9, Bezanilla et al., 1982).

Contractile Response to Single Voltage Clamp Steps

The contractile responses of a short fiber to voltage clamp pulses of different amplitudes and pulse durations of 50 or 100 ms are shown in Fig. 2A. For each pulse duration, the peak tension achieved, the time to peak tension, and the duration of the contraction increased as the internal potential was made progressively more positive. The peak tension ultimately saturated with increasing internal potentials. One noticeable feature of the responses is that for any given pulse duration, the rate of tension development for different internal potentials was the same within experimental variation, provided the peak tension generated was more than one-third of the maximum. In fact, for the responses illustrated in Fig. 2, except for those obtained with small depolarizations, the initial rate of tension development was the same for the two pulse durations shown. This point is better illustrated in Fig. 3B, which shows the results obtained with a different fiber.

When voltage clamp pulses of 10 ms duration were used, the behavior of the contractile response to increasing internal potentials was slightly different. For this pulse duration, both the peak tension and the initial rate of tension development increased as the internal potential was increased. The times to peak tension generally increased with increasing potential steps in a manner similar to that for the longer pulses shown in Fig. 2A. Thus, the main difference with 10-

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[1] It should be noted that although these measurements record the internal potential, they do not measure the membrane potential uniformity along the T-system, which is expected to deviate significantly at short times (Vergara and Bezanilla, 1981).

A

50 ms   IOO ms

IOO mV
1.3 kg/cm²
0.1s

B

0.1 s
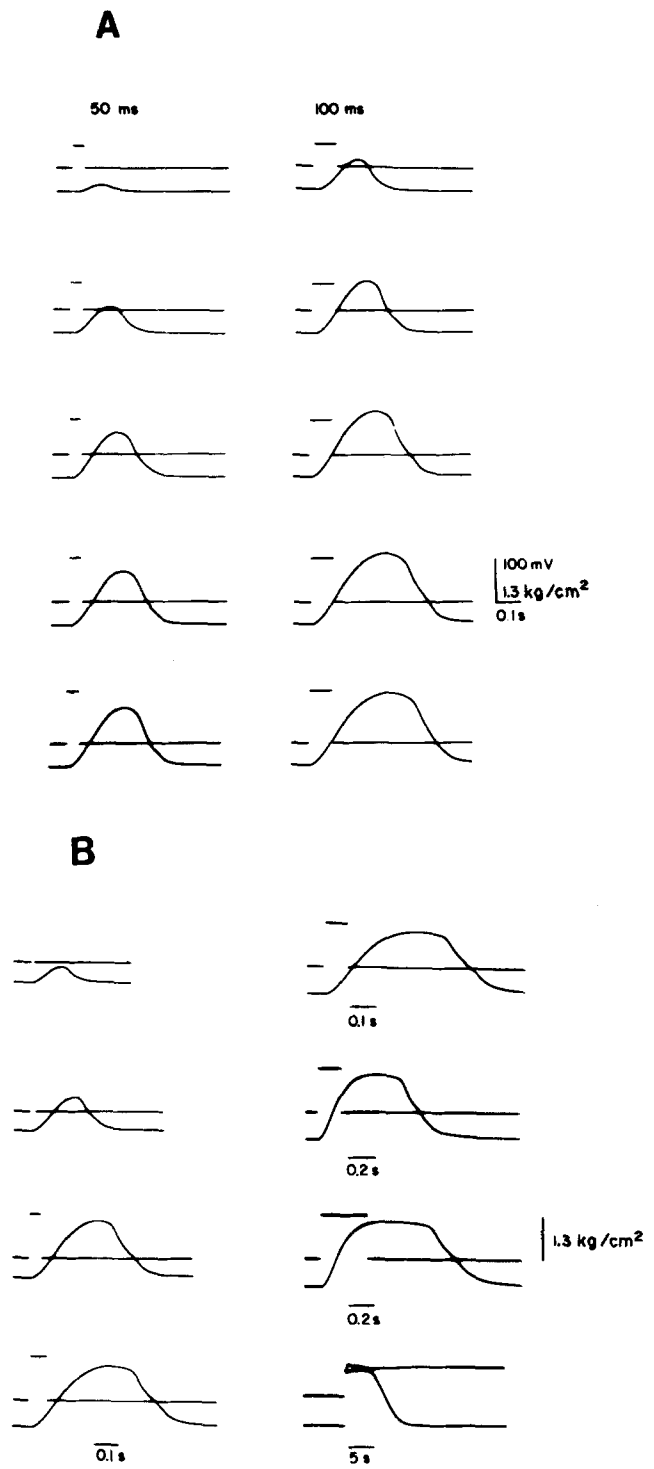
0.2 s

1.3 kg/cm²

0.2s

0.1 s   5 s

FIGURE 2.   Contractile responses to depolarizing pulses of different amplitude and 50 or 100 ms duration (A), and of fixed amplitude (100 mV) and varying duration (B). Holding potential, −90 mV. Temperature, 15°C.

ms pulses was that the initial rate of tension development increased with increasing internal potentials, over a wide range of potential values, reaching its maximal value at the same time as with longer pulses (~200 ms after the beginning of the pulse).

Fig. 2B shows the results of a run in which a pulse of 100 mV amplitude is progressively prolonged. For short pulses, force increases with pulse duration
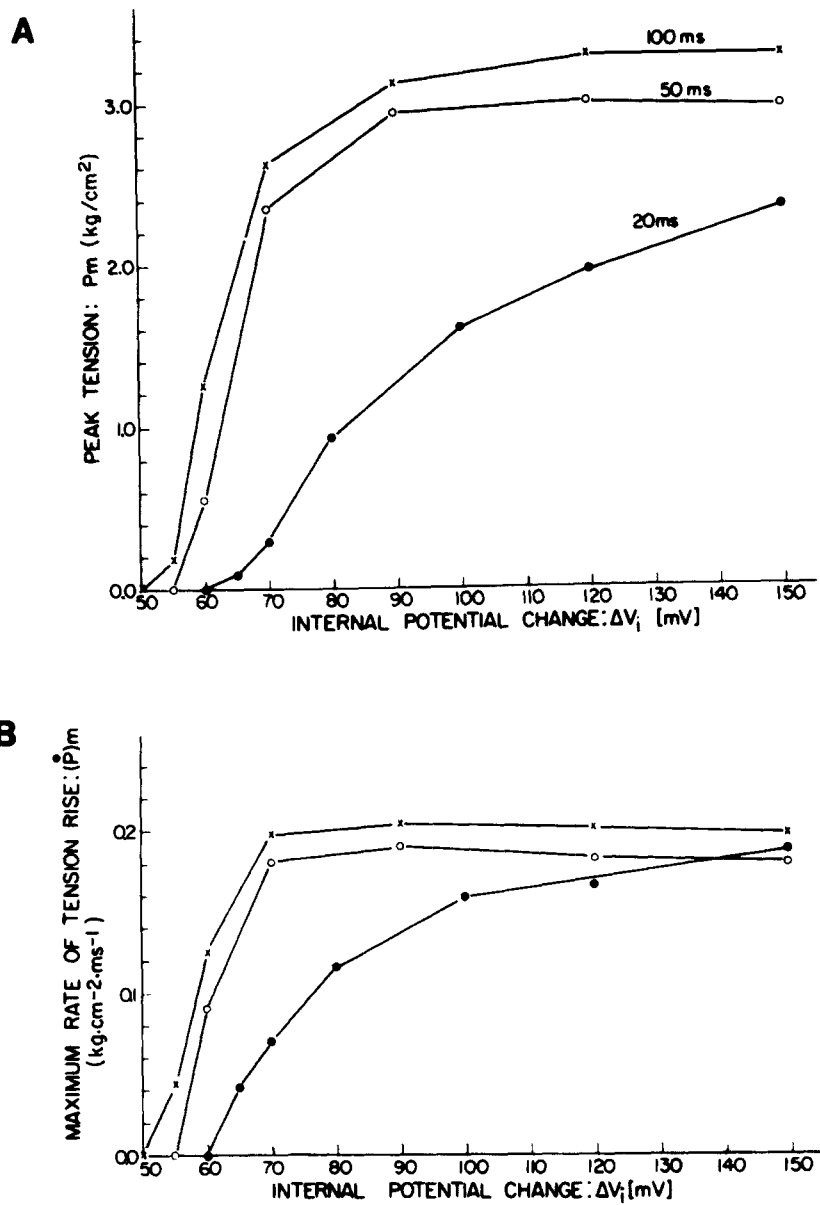
until it reaches its maximum value. Further prolongation of the pulse merely prolongs the contractile response.

Fig. 3 A shows the peak tension-potential relation for different pulse durations (20, 50, and 100 ms) derived from the data of another experiment. It is clear that the relation of peak tension to internal potential depends on pulse duration, especially for short pulses. The relations obtained differ in several respects. First, the threshold potential for mechanical activation is more positive for shorter pulses. This is an expression of the strength-duration curve for mechanical activation and agrees with the results of Adrian et al. (1969) and Costantin (1974). Second, the slope of the tension-potential curve increases as the pulse duration increases. The average maximum slope of the tension-potential curve for pulses of 50–100 ms duration was 8.4% of maximum tension per millivolt of depolarization. In general, for any given pulse duration, this slope varied considerably from fiber to fiber. Despite this variation, the maximum fiber tension saturated at about the same value with the longer pulses. For pulse durations of 20 ms or shorter, it was not always possible to reach the maximum value of tension obtained with longer pulses. However, as shown in Fig. 3 A, the peak tension was still increasing at the highest internal potentials achieved and appeared to be approaching the maximum value. Fig. 3 B shows that for pulses of 20, 50, and 100 ms, the maximum rate of tension development is reached with pulse amplitudes smaller than necessary for tension saturation. It also shows that with the 20-ms pulses the maximal rate of tension development is reached with substantially larger pulse amplitudes than with 50- and 100-ms pulses.

Tension-Time Integral

For pulses of both fixed duration with varying amplitude and fixed amplitude with varying duration, maximum tension is rapidly reached as the independent variable is increased. Although a further increase in the independent variable does not produce an increase in maximum tension, the total duration of the contraction generally continues to increase. For these circumstances, the increase in the duration of the contractile response presumably reflects an increase in the amount of activator available. This fact suggests that the area under the tension curve, the tension-time integral, provides a more reliable index of the relationship between the contractile response and the membrane potential-induced activator availability than does the peak tension developed.

FIGURE 3. (*opposite*) Tension-voltage relationships obtained from the same fiber with different pulse durations. Absolute tension P_m is expressed in units of kilograms times square centimeters. Voltage is expressed as the change ΔV_i in millivolts from a holding potential of -100 mV. The different behavior observed for the case of 20-ms pulses may in part be explained in terms of nonhomogeneous longitudinal control, which could determine the presence of series elastic elements constituted by nonactivated sarcomeres. Temperature, 14°C. (*B*) Relationships between the maximum rate of tension rise $(\dot{P})_m$ are expressed in units of kilograms per millisecond and voltage is expressed as the change ΔV_i in millivolts from a holding potential of -90 mV. Same experiment as shown in *A*.

The interpretation and use of the tension-time integral is considerably simplified by the simple time course of the relaxation phase and its insensitivity to pulse duration and amplitude. Fig. 4 gives a semilogarithmic plot of the decline of tension from two experimental series, one for fixed pulse amplitude with variable duration and another for fixed pulse duration with variable amplitude. From the results it is clear that the major portion of the relaxation phase is exponential and not appreciably affected by either pulse variable. This implies that differences in tension-time integral are due mainly to the initial magnitude of the mechanical response and the duration of the contraction and plateau phases.

With regard to voltage clamp pulses, the fact that both pulse amplitude and duration affect the contractile response indicates that neither variable by itself provides a complete measure of the electrical stimulus. Beyond threshold values, an increase in either pulse variable produces an increase in the tension-time integral. For simplicity, we have taken the product of the amplitude and duration as a measure of the electrical stimulus.

Fig. 5A shows how the tension-time integral varies as a function of the stimulus amplitude-duration product, for three fixed pulse durations and variable pulse amplitude. For each pulse duration, the tension-time integral was zero until a threshold value of the stimulus amplitude-duration product was reached. Beyond this value, the tension-time integral increased rapidly at first and then more slowly with a constant slope.

An important feature of this relation is that the tension-time integral continues to increase as the pulse amplitude is increased. If the tension-time integral is related to the amount of contractile activator liberated, this could mean that activator liberation does not saturate at membrane potentials as positive as +40 mV. Alternatively, it could mean that inward calcium currents flowing when the pulse is turned off (tail Ca^{++} currents) (Sánchez and Stefani, 1978; Horowicz and Schneider, 1981a) could add to the Ca^{++} liberated from the sarcoplasmic reticulum. This point was tested in several experiments carried out in the absence of external Ca^{++}, and with Ca^{++} replaced by Ni^{++}, which does not carry charge through the Ca^{++} channel and does not activate contraction by itself (Almers and Palade, 1981; Caputo, 1981). In all these experiments, a continuous increase of tension-time integral with pulse amplitude was observed, thus making the inward calcium current mechanism unlikely.

The linear increase in the tension-time integral with stimulus amplitude-duration product for a constant-duration pulse indicates that for every millivolt increase in pulse amplitude, there is a proportionate increase in the tension-time integral for internal potentials between −20 and +50 mV (see Fig. 5A). Furthermore, since the slopes of the linear portions of the individual curves are the same within experimental variation, this implies that equal increments in the stimulus amplitude-duration product produced the same increment in the tension-time integral, independent of the pulse duration for internal potentials between −20 and +50 mV. For example, a 5-mV increment in amplitude for a 20-ms pulse produced about the same increment in the tension-time integral as did either a 2.0-mV increment for a 50-ms pulse or a 1-mV increment for a 100-ms pulse.
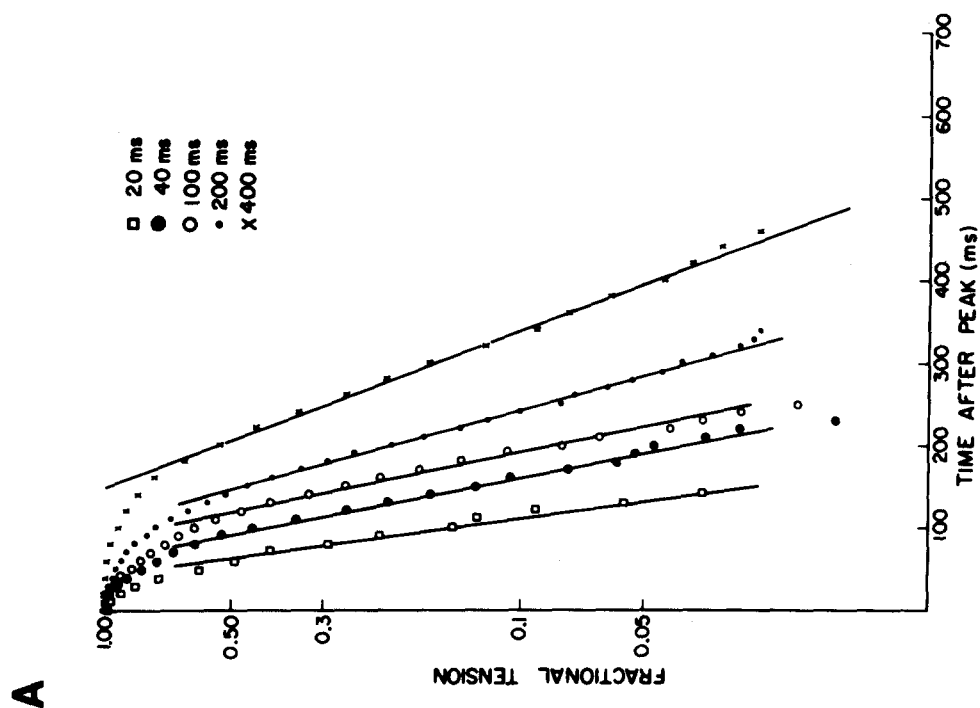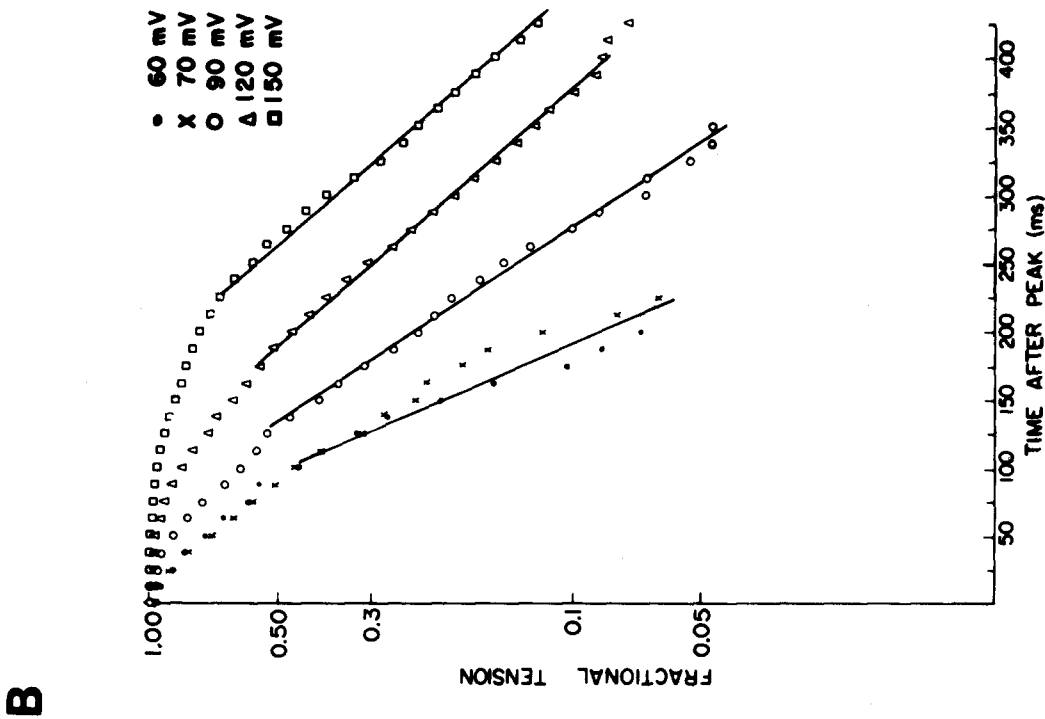
FIGURE 4. Semilogarithmic plots of fractional tension vs. time after peak tension, obtained with two different fibers. In A the pulse amplitude was 80 mV and the holding potential was −90 mV, and in B the pulse duration was 100 ms and the holding potential was −100 mV. In both cases a sizeable fraction of the relaxation phase follows an exponential decay whose time constant appears to be independent on the pulse duration (A) and pulse amplitude (B). Temperature (A and B), 12.5 °C.
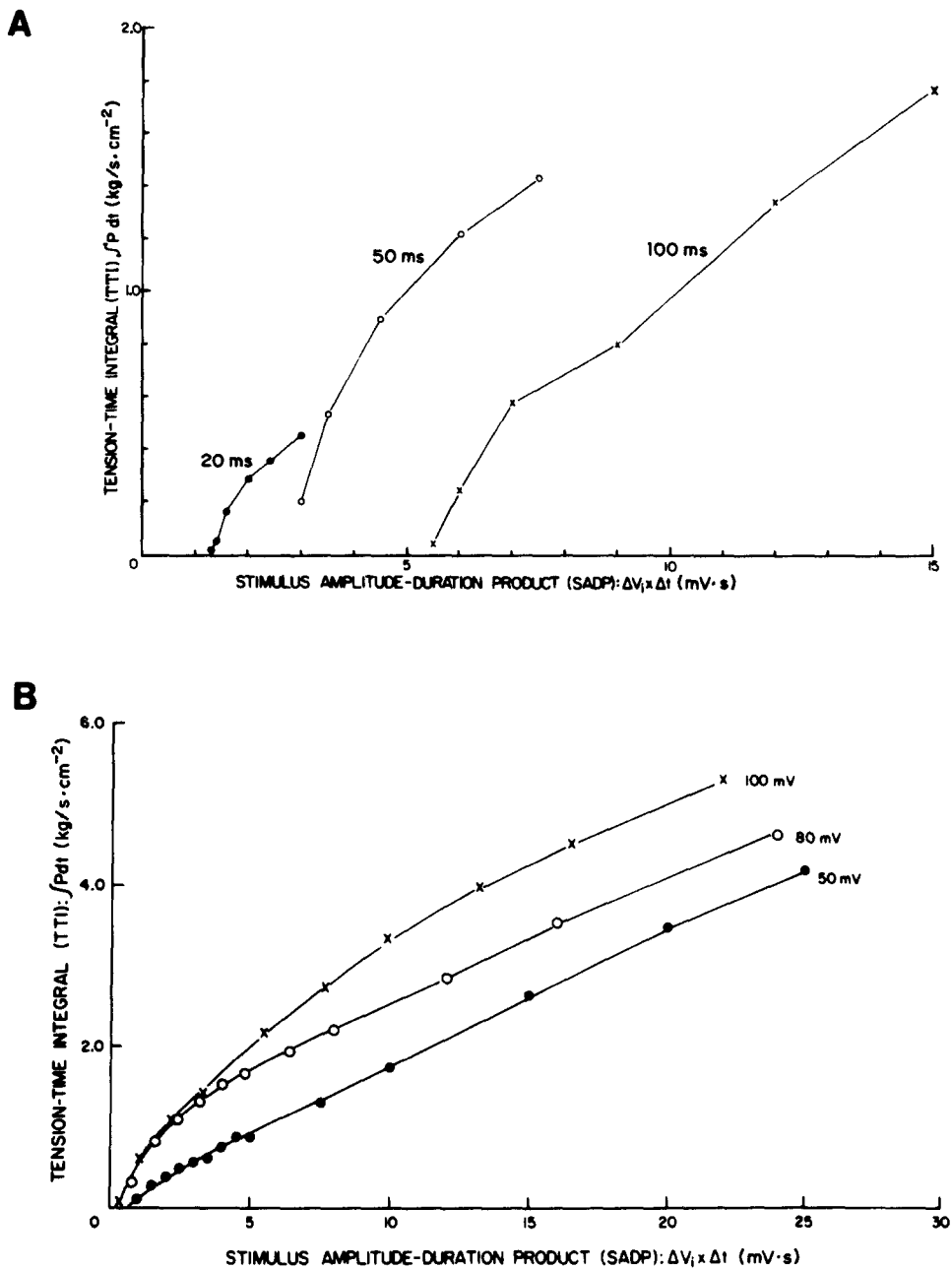
FIGURE 5.   Relationships between the tension-time integral ($\int Pdt$) expressed in kilograms per second per square centimeter and the stimulus amplitude duration product. The curves in A were obtained with three fixed pulse durations (20, 50, and 100 ms), while the pulse amplitude was varied. The curves in B were obtained with three fixed pulse amplitudes (50, 80, and 100 mV) while the pulse duration was varied. Holding potential, −90 mV. Temperature, 12°C.

Fig. 5*B* illustrates, in another fiber, the variation of the tension-time integral as a function of the stimulus amplitude-duration product for three fixed-amplitude pulses as pulse duration was varied. As in Fig. 5*A*, the tension-time integral is zero until a threshold value of stimulus amplitude-duration product was reached. Since the pulse amplitudes chosen were well up on the strength-duration curve (i.e., well above "rheobase"), the threshold stimulus amplitude-duration products are much closer together and have relatively low values compared with the pulse configurations used in the experiment depicted in Fig. 5*A*. The tension-time integral increases rapidly as the stimulus amplitude-duration product is increased just above the threshold values and then becomes linear with a smaller slope at higher values of the stimulus amplitude-duration product. The pulse durations beyond which the curves became linear varied between 30 and 70 ms for the three curves. As was the case with constant-duration pulses, the linear portions of the curves have essentially the same slope, which indicates that equal increments in tension-time integrals were produced by equal increments of the stimulus amplitude-duration product regardless of pulse amplitude. The average slope of the linear portions of the curves from the experiment of Fig. 5*B* is 0.063 kg/s·cm^{-2} of tension-time integral per millivolt per second of stimulus amplitude-duration product; for the experiment of Fig. 5*A*, the average slope is 0.16 in the same units.

Effect of Conditioning Pulses

It has been shown that subthreshold conditioning pulses can, under certain conditions, potentiate the contractile response elicited by a test pulse (Bezanilla et al., 1971). Fig. 6 confirms this observation. In records *a*, *d*, *c*, and *f*, the effect of doubling the duration of a pulse of a given amplitude is shown. Records *b* and *e* show the effect of two identical pulses separated by a complete (*b*) or partial repolarization during the interval. Finally, records *g*–*i* show that a pulse, which by itself induces no tension (record *i*), is effective in inducing a potentiation when applied either on the leading (*h*) or the trailing (*g*) edge of a test pulse (*a* and *c*).

In order to better define the effects produced by a conditioning pulse, test pulses were chosen in the region where the tension-time integral increases linearly with stimulus amplitude-duration product. The results obtained in one such experiment are shown in Fig. 7*A*. In this experiment, both the conditioning and test pulses had a duration of 50 ms. The amplitude of the test pulse was 100 mV, while the amplitude of the conditioning pulse was variable. At the start, the mechanical response to the conditioning pulse was determined. The crosses and the curve through them depict the tension-time integral produced by the conditioning pulse alone as a function of the amplitude-duration product. The curve is similar to that of the experiments illustrated in Figs. 4 and 7*B*.

Throughout the experiment, the fiber was periodically stimulated by a test pulse alone or by a test pulse preceded by a conditioning pulse of the same amplitude as the test pulse to check the responsiveness of the fiber as the experiment proceeded. The results of these checks are plotted as filled circles. In effect, this procedure is equivalent to altering a stimulating pulse of constant amplitude by varying its duration. It is clear from the results shown in Fig. 7*A*
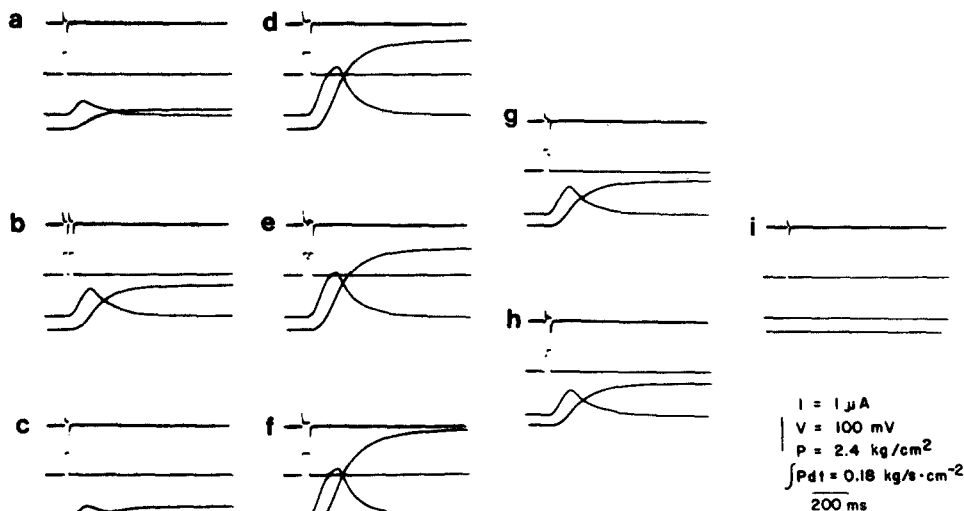
I = 1 μA
V = 100 mV
P = 2.4 kg/cm²
∫Pdt = 0.18 kg/s·cm⁻²
200 ms

FIGURE 6. Effect of depolarizing pulse on contractile response and tension-time integral. In each record the upper trace represents the current I, the second represents the voltage V, and the lower two traces represent tension and tension-time integral, $\int Pdt$, respectively. The effect of temporally adding two identical pulses (70 mV, 25 ms) is shown in records a, d, c, and f. Records a, c, g, h, and i show the effect of adding a subthreshold pulse (i) at the leading (h) or trailing (g) edge of a test pulse (70 mV, 25 ms) (a and c). Experiments of this type are further described in Fig. 7. Records b and e show the effect of interposing a brief interval with total or partial repolarization between two identical pulses. Holding potential, −90 mV. Temperature, 15°C. Further experiments of this type are described in Figs. 8 and 9.

FIGURE 7. (*opposite*) Effect of double-pulse pattern on the relationship between the tension-time integral ($\int Pdt$), expressed as in Fig. 5, and the stimulus amplitude-duration product. In A, a test pulse of 100 mV amplitude and 50 ms duration was preceded by a 50-ms pulse of variable amplitude. The curve drawn through the crosses represents the tension-time integral vs. the pulse amplitude-duration product obtained with the test pulse alone. The filled circles represent the values of $\int Pdt$ obtained with the 50-ms, 100-mV single pulse or the 100-ms, 100-mV double pulse. The curve through the open circles shows the effect of adding the extra tension-time integral associated with the conditioning pulse to the value obtained with the 50-ms, 100-mV test pulse. The curve through the triangles shows the results obtained with the test pulse alone (crosses) starting from the level obtained with the 50-ms, 100-mV configuration. Holding potential, −90 mV. Temperature, 15°C. In part B the variable-amplitude, fixed-duration pulse, was applied at the trailing edge of the 50-ms, 100-mV fixed conditioning pulse. The curves through the crosses, the open circles, and the triangles have the same meaning as those shown in A. The open squares represent experimental points obtained by gradually increasing the duration of the conditioning pulse to twice its normal value of 50 ms. Holding potential, −90 mV. Temperature, 15°C.

that a line drawn through the averages for each of these two pulses of constant amplitude but different durations has a slope which is the same as that of the linear portion of the conditioning pulse series (i.e., the curve through the last four crosses), in which the duration was kept constant but the amplitude was varied. This reinforces the conclusion of the previous section that the slopes of the tension-time integral vs. stimulus amplitude-duration plots in the linear

A

B
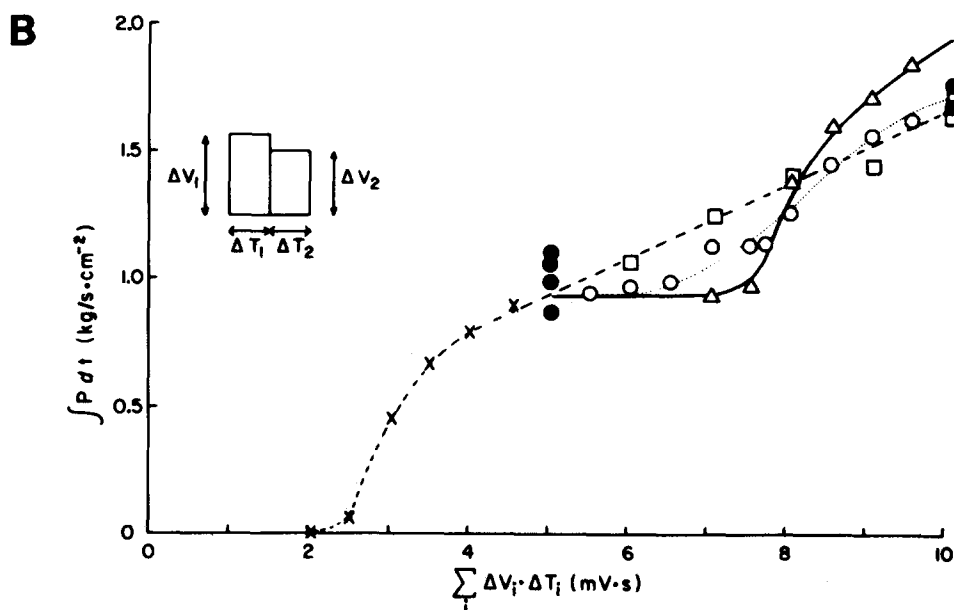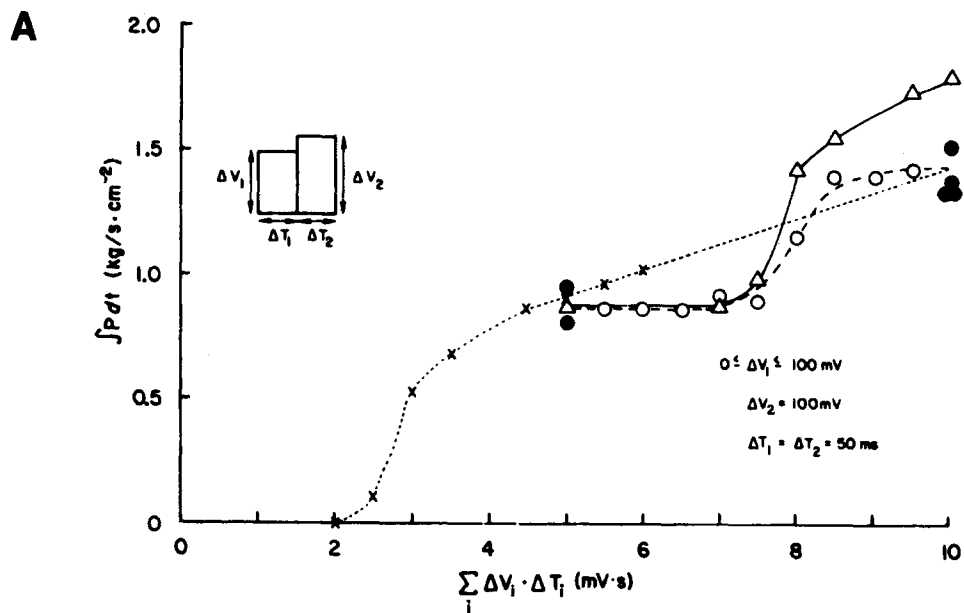
regions are the same for both constant-duration and constant-amplitude pulses.

The open circles and the dashed curve give the tension-time integral of the response as a function of the sum of the amplitude-duration products of both the conditioning and test pulses. From the results it is clear that as the conditioning pulse amplitude is gradually increased from 0 mV, there is at first no effect on the contractile response obtained with the test pulse alone. Beyond a certain conditioning pulse amplitude, however, the tension-time integral increases monotonically to the level produced by a single pulse of 100 mV amplitude and 100 ms duration. The threshold value of amplitude beyond which the conditioning pulse begins to cause an increase in the tension-time integral above the value obtained with the test pulse alone is not measurably different, in this type of experiment, from the threshold value that produces a contractile response when the conditioning pulse is applied alone. This indicates that when the test pulse produces a response in which the peak tension is close to the maximum and recruitment of contractile elements is no longer a contributing factor, subthreshold conditioning pulses do not appear to produce any detectable effect on the mechanical response as measured by the tension-time integral. For comparison, the open triangles and the solid curve through them have been calculated by adding the average tension-time integral from the test pulse alone to the tension-time integral obtained for the series in which only the conditioning pulses were applied, and then plotting these values as a function of the sum of the amplitude-duration products for the two separate pulses. It is clear that beyond the threshold amplitude of the conditioning pulse, the increment in the tension-time integral produced when both pulses were applied together was smaller than the calculated increment in the tension-time integral when the pulses were applied separately. This indicates that in the presence of the conditioning pulse, the activation effectiveness of the test pulse, as measured by the increment in tension-time integral, is reduced for all amplitudes of the conditioning pulse beyond threshold.

Experiments were also performed in which the variable-amplitude pulse constituted the trailing edge of the total pulse rather than the leading edge, as in the previous experiments. Fig. 7B shows the result from one such experiment, plotted in the same way as Fig. 7A. In this case, however, with a fixed-amplitude, fixed-duration conditioning pulse, the activation effectiveness of a subsequent variable-amplitude, fixed-duration test pulse is increased for a range of amplitudes from slightly below threshold to slightly above threshold as compared with when the test pulse is applied alone. This is indicated by the fact that in this range the open circles are above the open triangles. When the amplitude of the second pulse is more than slightly greater than threshold, its activation effectiveness is decreased, as indicated by the fact that the open circles fall below the open triangles for the larger pulse amplitudes.

Effects of Pulse Separation

In Fig. 6, it was shown that a brief separation between two identical pulses was effective in reducing the peak tension and tension-time integral relative to application of the pulses with no separation. In several experiments of this type,
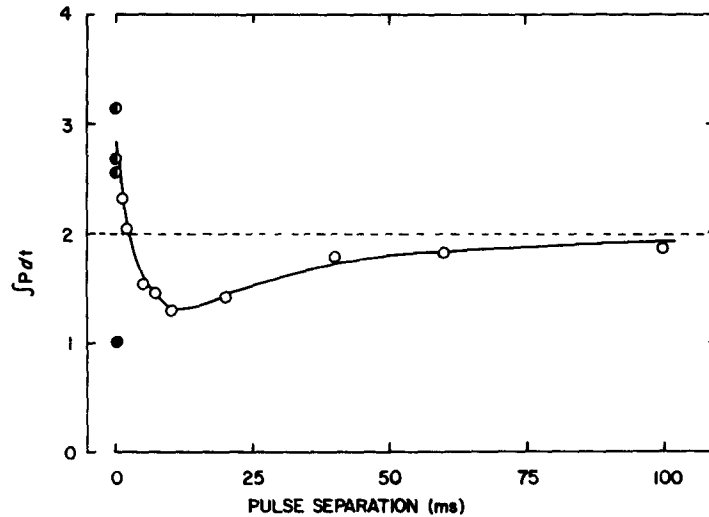
FIGURE 8. Effect of pulse separation on the tension-time integral of the response to two identical pulses of 20 ms duration and 50 mV amplitude. Holding potential, −90 mV. The black dots represent the ∫Pdt obtained with one pulse alone. The half-filled circles represent the values obtained with a pulse of twice the duration of the first pulse (i.e., two identical pulses applied consecutively). The three values were obtained at different times during the experiment and are indicative of changes of the fiber contractile behavior. The empty circles show the values of ∫Pdt when the two pulses were separated by different time intervals. Temperature, 12°C.
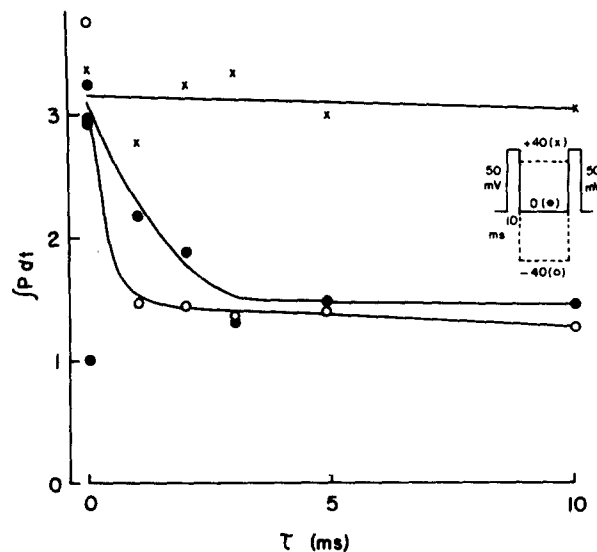

FIGURE 9. Effect of the membrane potential level during pulse separation on the tension-time integral of responses to two pulses applied with different time intervals. The inset shows the pulse protocol, with the symbols indicating the value of the membrane potential changes from a holding potential of 100 mV. Thus, the open circles, filled circles, and crosses indicate interpulse potentials of −140, −100, and −60 mV, respectively. Temperature, 12°C.

it was found that as the time separation between pulses was increased, the tension-time integral of the response decreased for brief intervals and then increased at somewhat longer intervals. Eventually, for long intervals, the two individual responses became nearly separate. Surprisingly, in many fibers, the initial minimum in the tension-time integral from a double pulse was well below twice the integral from a single pulse. One such experiment is shown in Fig. 8. The minimum tension-time integral obtained with a 10-ms interruption was only 30% greater than that produced by a single pulse. Beyond this time, the normalized tension-time integral increased monotonically to a final level equivalent to the sum of two separate responses. The fact that the normalized tension-time integral of the response to two pulses fell below a value of two means that the second pulse was less effective in activating the contractile system than if it had been applied alone. In other experiments, it was observed that the dip in the tension-time integral at ~10 ms gradually disappeared as the amplitude of the pulses was increased to 100 mV.

Additional experiments were performed to determine how the membrane potential during the interpulse interval affected the tension-time integral of the response. Fig. 9 shows the results of one such experiment. When the fiber was repolarized to the holding potential during the interpulse interval, a rapid decay of the contractile response occurred after the first pulse (filled circles). This decay was greater and more rapid when the fiber was hyperpolarized by 40 mV during the period between the pulses. By contrast, the decay in response could be abolished by maintaining the fiber at a depolarized level, which by itself was subthreshold, during the interpulse interval.

DISCUSSION

Contractile activation is a graded phenomenon under tight control by the membrane potential (Hodgkin and Horowicz, 1960). Although the application of the two-microelectrode voltage clamp technique to short muscle fibers allows measurement of isometric tension, this approach suffers from some theoretical and experimental limitations (Bezanilla et al., 1982). Ideally, one aim of the experimental design in a study of the relations between excitation and contraction is to measure the response of the contractile elements to appropriate stimulation of the excitatory element. For intact striated muscle fibers, the unit contractile element is the myofibrillar half-sarcomere, and the unit excitatory element is the plasma membrane of the transverse tubule associated with the nearest triad. An important experimental limitation is that an intact fiber is composed of many contractile and excitatory elements organized in a distributed structure. In such an extended structure, which is best described for electrical purposes as a distributed network, there are limits to the speed and uniformity with which the potential can be controlled both radially and longitudinally using two internal microelectrodes (Bezanilla et al., 1982).

In this work, two stimulus parameters have been varied: amplitude and duration. As either stimulus parameter is increased, threshold is reached in some region of the muscle fiber. Further increase not only produces increased output in the responding contractile element but also recruits additional contractile

elements into the responding pool. Certain features of the activation mechanism aid in discerning when all contractile elements have been recruited. It was found, for example, that the peak rate of tension development reached a constant, maximum value for stimuli not far above threshold. This occurred in a potential range where both the peak tension and the tension-time integral were increasing monotonically. In most cases the peak tension also saturates at a constant maximum value for stimuli somewhat greater than required to saturate the peak rate of tension development.

One can assume that when the peak tension developed has reached its maximum value, all the contractile elements have been recruited. Whenever the stimulus variables are in the range where the maximum peak tension is achieved, the peak rate of tension development has already saturated at its maximum value. Therefore, this implies that when the peak rate of tension development has reached its maximum value, all contractile elements are being activated and recruitment is complete. Since constant values of maximum tension and peak rate of tension development indicate that recruitment is no longer taking place, the increase in tension-time integral as pulses increase indicates that more activator is available and that there is no contribution of recruitment of new contractile elements to the response, which agrees with the conclusion of Costantin (1974). As tension approaches its maximum value, increases in activator release are no longer reflected in increases in peak tension, and at this point the tension-time integral becomes a more adequate measure of the activator release process.

In this work, the term "activator" is being used to refer to the fraction of calcium that binds to troponin after being released from the sarcoplasmic reticulum. Thus, "activator release" is not meant to be the same as "calcium release," but only an indirect and approximate measure of it.

From the results shown in Figs. 5 and 7, it appears that above the threshold region, the tension-time integral value increases linearly with the stimulus amplitude-duration product, regardless of whether the product is increased by increasing the amplitude or the duration. Furthermore, in this linear region it appears that the slope of the plots of tension-time integral vs. stimulus amplitude-duration product were the same regardless of the independent variable and had a value of ~0.1 $kg/s \cdot cm^{-2}$ of tension-time integral per $mV \cdot s$ of stimulus amplitude-duration product. The continuous increase of the tension-time integral up to membrane potentials values of +50 mV indicates that activator calcium release may still be increasing at positive potential values, which is in agreement with results obtained using metallochromic calcium indicators (Kovács et al., 1979; Palade and Vergara, 1982). It is also noteworthy, in this regard, that in the recently reported experiments of Miledi et al. (1983b), the peak response of the metallochromic calcium indicator arsenazo III to 20-ms pulses continues to increase as the pulse potential is increased up to values of +80 mV in some fibers. These results are not incompatible with the idea that intramembrane charge movement is the mechanism that provides the voltage sensitivity to calcium release, since total charge movement is not saturated at membrane potentials of +20 mV (Horowicz and Schneider, 1981a, b).

Recent evidence obtained using arsenazo III indicates that the potentiation of the contractile response caused by a subthreshold conditioning pulse is not accompanied by a subthreshold release of calcium (Miledi et al., 1981). This result suggests that the potentiation produced by the second pulse is mediated by an increased calcium release caused by facilitation of the coupling mechanism or alternatively by improved penetration of the signal into the T-system.

An important result related to such a potentiating effect is the demonstration that intramembrane charge movement may occur during a conditioning pulse below the threshold for contraction (Horowicz and Schneider, 1981b), and that when this occurs the duration of the test pulse necessary to reach threshold is shortened, while the total charge moved at contraction threshold remains the same. The effect of the prepulse is to allow part of the total threshold charge to be moved prior to the test pulse. The results presented in this paper are therefore compatible with the idea that charge movement, or some component of it, provides the voltage sensitivity to contractile activation.

Recently, it has been shown that mechanical inhibition may follow a brief depolarization in mammalian or amphibian muscle fibers when the contractile threshold is determined with very short (2 ms) test pulses (Dulhunty, 1982). In addition, it is known that when a muscle is stimulated with a train of action potentials, the intracellular Ca^{++} response to a second and subsequent action potentials is markedly less than the response to the first action potential (Blinks et al., 1978; Miledi et al., 1983a). Both these effects are likely to be related to the decreased mechanical effectiveness of the second of two depolarizing pulses in the experiments reported here.

In summary, it can be said that the relation between peak tension and depolarization amplitude reflects the involvement of several factors. First, the duration of the stimulus determines the pulse amplitude required to reach threshold. Beyond threshold, the increase of tension with increasing depolarization reflects recruitment of additional contractile elements, as well as increases in the mechanical output of those elements already responding. At some level of depolarization, recruitment of excitatory elements into the responding pool is complete, and further increases in contractile output are due solely to further cross-bridge recruitment and activation produced by increased activator availability. Finally, when all the cross-bridges available for tension development are engaged and the mechanical apparatus reaches saturation, further increases in the availability of activator mainly prolong the duration of the contractile response. As a consequence, the relation between peak tension and depolarization is of limited usefulness as a measure of the relation between activator release and depolarization at the level of the triad. The relationship between tension-time integral and depolarization is a better indicator for calcium release, but it is still unsuitable for identifying putative components of charge movement directly related to activation. The presence of several Ca binding sites other than troponin in the myoplasm makes it difficult to interpret directly the tension-time integral in terms of "calcium release" from the sarcoplasmic reticulum. Measurements of Ca release with metallocromic indicators, although they also suffer from these limitations (Baylor et al., 1983; Kovács et al., 1983), would circumvent the

distortions introduced by recruitment and saturation of mechanical elements but would not eliminate the distributed nature of the transverse tubular system and the triads in intact fibers and the progressive activation of triads for a range of depolarizations beyond threshold. This is the most likely explanation for the steeper than expected relation between peak arsenazo response and depolarization obtained near threshold in the experiments of Miledi et al. (1983*b*).

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