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CALCIUM TRANSIENTS IN FROG SKELETAL MUSCLE FIBRES FOLLOWING CONDITIONING STIMULI

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SUMMARY

1. Intracellular Ca^{2+} transients were recorded from frog twitch muscle fibres, using arsenazo III as a Ca^{2+} monitor. When fibres were stimulated by two action potentials, the arsenazo signal to the second stimulus was smaller than the first, for stimulus intervals of up to several seconds.

2. The recovery of the amplitude of the second response followed two exponential time courses; a fast one with a time constant of about 70 msec giving recovery to about 90% of the control value, followed by a slow recovery to 100%, with a time constant of about 12 sec (at 10 °C).

3. The time constant of the fast recovery component was strongly temperature-dependent, with a Q_{10} of approximately 2·7, whilst the Q_{10} of the slow component was about 1·4.

4. Removal of Ca^{2+} in the bathing medium lengthened the time constant of the slow recovery component by a factor of three, but had little effect on the fast recovery component. The lengthening of the slow component was not reversed by addition of Mg^{2+}, but Sr^{2+} ions could substitute for Ca^{2+}.

5. The influence of membrane potential on the recovery time-course was investigated after blocking action potentials with tetrodotoxin, using a voltage clamp to control membrane potential. Paired depolarizing stimuli were used, with the potential held to either low (−60 or −80 mV) or high (−110 or −140 mV) potentials between stimuli. No differences were apparent in either the fast or slow recovery components at these holding potentials.

6. The arsenazo response elicited by an action potential following a conditioning tetanus was reduced in size even more strongly than following a single action potential. The time course of recovery of the response following a tetanus again comprised two exponential components. After a 20 Hz tetanus for 0·5 sec, the fast component had a time constant of about 400 msec, and gave a recovery to about 60% of the control value. Subsequent recovery to 100% occurred with a time constant of about 12 sec.

7. The time constant of the fast recovery component increased markedly with increasing frequency or duration of the conditioning tetanus. The time constant of the slow component was not appreciably altered by conditioning tetani varying between one impulse and sixty impulses. However, the reduction in response size due

to the slow component, extrapolated to zero stimulus interval, increased with increasing number of impulses in the tetanus.

8. The time constant of the fast recovery component corresponded closely with the decay time constant of the arsenazo response to the conditioning stimulus. This correspondence held over a nearly fifty-fold range of time constants, and for two different conditions which affected the decay time constant (temperature, and frequency of tetanic stimulation).

9. The decay time constant of the arsenazo response elicited by an action potential was slowed by a preceding impulse or tetanus. Following a 20 Hz tetanus for 0·5 sec, recovery of the half decay time appeared to follow an exponential time course, with a time constant of about 12 sec.

10. These results suggest that the fast recovery component reflects the re-filling of release stores in the sarcoplasmic reticulum by Ca^{2+} ions taken up from the cytoplasm. The origin of the slow component is less clear, but it may arise from inactivation of the excitation–contraction (e–c) coupling process between T-tubule depolarization and Ca^{2+} release from the sarcoplasmic reticulum.

INTRODUCTION

It has been known for many years that the force developed by skeletal muscle fibres decreases during a prolonged depolarization, and test contractures induced at different times after repolarization show a slow recovery (Hodgkin & Horowicz, 1960). More recently, with the development of techniques for monitoring intracellular Ca^{2+}, it has been possible to show that comparable changes are seen in the release of Ca^{2+} ions from the sarcoplasmic reticulum (Miledi, Parker & Schalow, 1979, 1981; Miledi, Parker & Zhu, 1982; Blinks, Rüdel & Taylor, 1978; Kovács, Rios & Schneider, 1979). For example, when a twitch fibre is stimulated repetitively to give a train of action potentials, the free Ca^{2+} change induced by successive action potentials shows a progressive decrease (Blinks *et al.* 1978; Miledi *et al.* 1982), and after the end of stimulation a considerable time is required before the response to a test action potential recovers to the rested-state level.

Two general hypotheses have been proposed to account for the depression and subsequent recovery of response size following tetanic stimulation or K^+ depolarization. One is that the store of Ca^{2+} ions available for release from the sarcoplasmic reticulum (s.r.) becomes depleted, and is only slowly re-filled (Winegrad, 1968, 1970; Connolly, Gough & Winegrad, 1971; Hodgkin & Horowicz, 1960; Blinks *et al.* 1978). Another possibility is that some stage in the excitation–contraction (e–c) coupling process between T-tubule depolarization and Ca^{2+} release from the s.r. becomes inactivated during depolarization, and recovers slowly (Frankenhaeuser & Lännergren, 1967; Caputo, 1972, 1981; Caputo & De Bolaños, 1979). Inactivation of intra-membrane charge movement has been proposed as a possible basis for this latter hypothesis (Chandler, Rakowski & Schneider, 1976; Adrian, Chandler & Rakowski, 1976; Rakowski, 1978; Kovács *et al.* 1979).

This paper presents a quantitative analysis of the recovery of the intracellular Ca^{2+} transient following conditioning action potentials or depolarizations, using arsenazo III as an intracellular Ca^{2+} monitor. The linearity of the arsenazo response size with

free [Ca^{2+}] (Miledi, Parker & Schalow, 1980) makes this technique particularly suitable, and its sensitivity is such that changes in response size of the order of 1 % can be resolved.

We find that the recovery time course of the Ca^{2+} transient follows two exponential components. The faster of these probably reflects the rapid re-filling of Ca^{2+} stores in the s.r., whilst the slower may arise from inactivation of the e–c coupling process.

METHODS

The preparation and recording techniques were as described previously (Miledi *et al.* 1982).

RESULTS

Arsenazo responses to paired stimuli

Previous experiments have shown that Ca^{2+} transients elicited by successive impulses during repetitive stimulation exhibit a gradual decrease or 'descending staircase' (Blinks *et al.* 1978; Miledi *et al.* 1982). To examine the factors which might be responsible for this, we began by studying the responses to paired action potentials delivered at different intervals.

Fig. 1 illustrates action potentials and corresponding arsenazo responses recorded at three different sweep speeds. Each frame shows the arsenazo signal to a single action potential, with superimposed responses to a second action potential at different intervals. At short stimulus intervals (less than a few hundred milliseconds) the arsenazo response to the second action potential occurs during the falling phase of the first response, but it is clear that the increase in Ca^{2+} level due to the second action potential is considerably smaller than that elicited by the first (Fig. 1*A*, *B*). With increasing stimulus interval, the size of the second response recovers rapidly for intervals between about 20 and 100 msec (Fig. 1*B*), but is still only about 90 % of the initial response after 1 sec. The final recovery back to 100 % takes some tens of seconds (Fig. 1*C*).

A possible explanation for the reduction in size of the second arsenazo signal is that it results from a reduction in action potential amplitude or duration. In the case of short stimulus intervals (< 500 msec) this is clearly not the case. For example, in Fig. 1*A*, the peak overshoot is less than 3 mV smaller for the second action potential compared to the first, and this would be expected to decrease the size of the arsenazo signal by only about 3 % (R. Miledi, I. Parker & P. H. Zhu, unpublished observations). Also, at intervals of a few tens of milliseconds, the second action potential occurs during the positive after-potential following the first stimulus (Fig. 1*A*). This would tend to slightly increase the response size (Miledi, Nakajima, Parker & Takahashi, 1981). At stimulus intervals longer than 1 sec, the reduction in size of the second response is small (maximum of 10 % reduction), and any changes in action potential parameters might have a more significant effect. To check this, a separate experiment was performed to measure the overshoot and duration (at 0 mV) of paired action potentials elicited at a 2 sec interval. Comparing the second action potential with the first, the mean overshoot was reduced by 0·5 mV, and the duration increased by

0·05 msec (results from four fibres). The mean overshoot for the first action potential was +23 mV, and the duration 1·6 msec. Thus, the decrease in amplitude would be expected to cause only a negligible decrease in arsenazo signal (< 1 %), whilst the

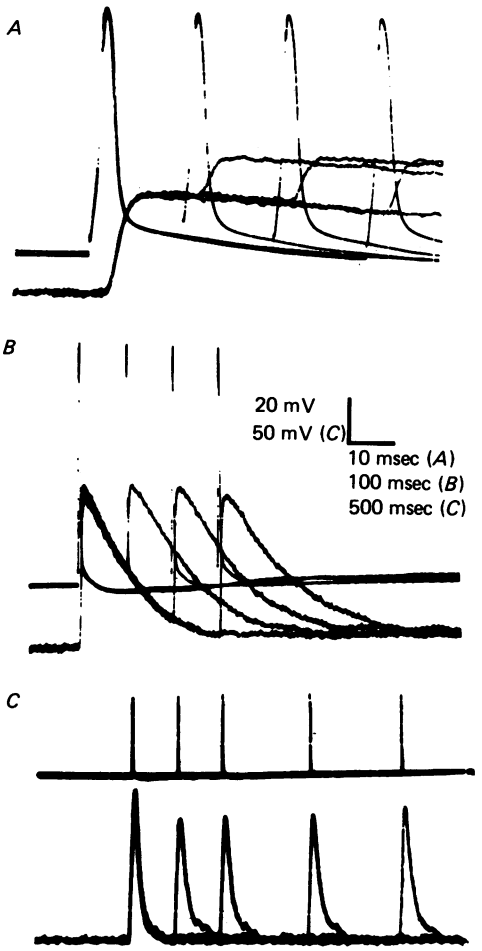

Fig. 1. Arsenazo responses to paired action potentials, illustrating the depression of the second response. In each record the upper trace is membrane potential and the lower light transmission at 650–700 nm. Four (*A* and *B*) or five (*C*) superimposed sweeps are included in each record to show the response to the first impulse alone, and to paired stimuli at different intervals. Action potentials were initiated by passing 2 msec duration depolarizing pulses through the dye pipette. A period of 1 min was allowed between sweeps. Time calibrations are: 10 msec, (*A*); 100 msec, (*B*) and 500 msec, (*C*). ΔI calibrations are: 0·008, (*A*); 0·007, (*B*); and 0·002, (*C*). Temperature 9–11 °C. Note that in all cases the increment in the arsenazo signal elicited by the second impulse is smaller than the first, even though the peak heights may appear similar (e.g. in *B*).

increase in duration is slight (~ 3 %), and would tend to increase the size of the second response.

The time course of recovery of the second response is plotted in Fig. 2, which shows pooled data from six fibres. In this and subsequent Figures, the peak size of the second

response is measured after subtracting any residual response from the first action potential, and is expressed as a percentage of the peak response to the first impulse of the pair. An interval of 60 sec was allowed for recovery between each pair of stimuli. Some fibres showed a gradual change in response size during the recording period,

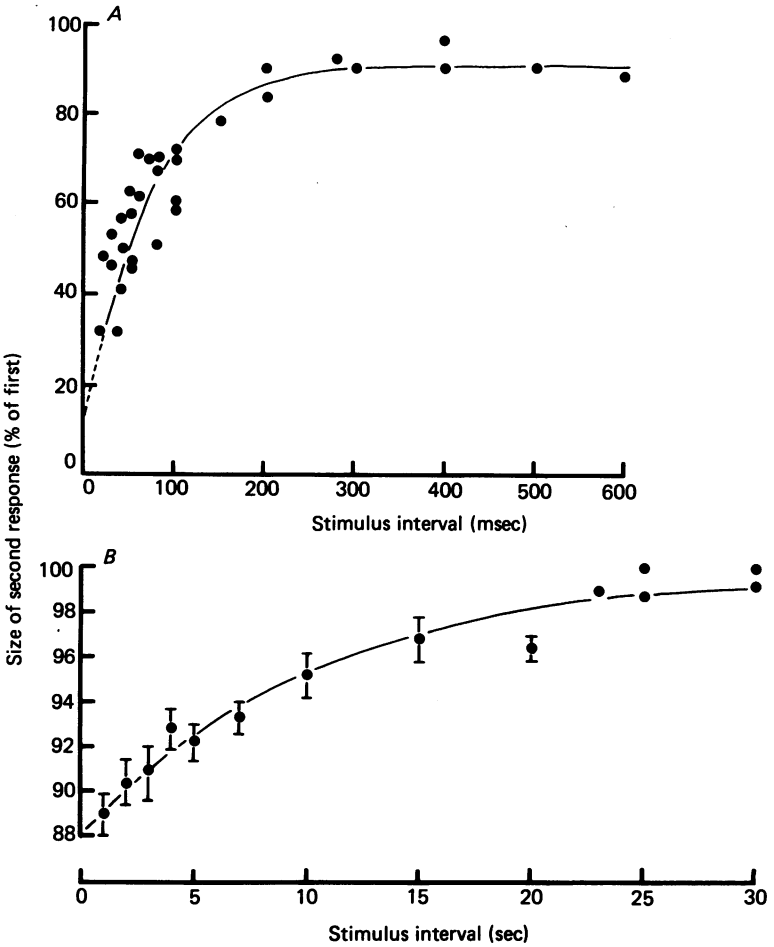

Fig. 2. Time course of recovery of the arsenazo signal elicited by the second of a pair of action potentials. In each graph the ordinate shows the interval between stimuli, and the abscissa the peak size of the second response as a percentage of the first. Data were obtained from six fibres and are plotted on different time scales in *A* and *B*. An interval of 60 sec was allowed between tests. Points with error bars indicate mean and S.E. of the mean from at least five measurements; points without bars are single measurements. Curves fitted to the data are exponentials with time constants of 77 msec (*A*) and 11 sec (*B*). Temperature, 9–11 °C.

probably because of changes in intracellular dye concentration. In the few cases where this effect would have introduced a significant error, a correction was made by estimating the control response size at the time of the test stimulus, by interpolating from the two bracketing control responses.

Recovery of the second response occurs with two quite distinct time courses. At

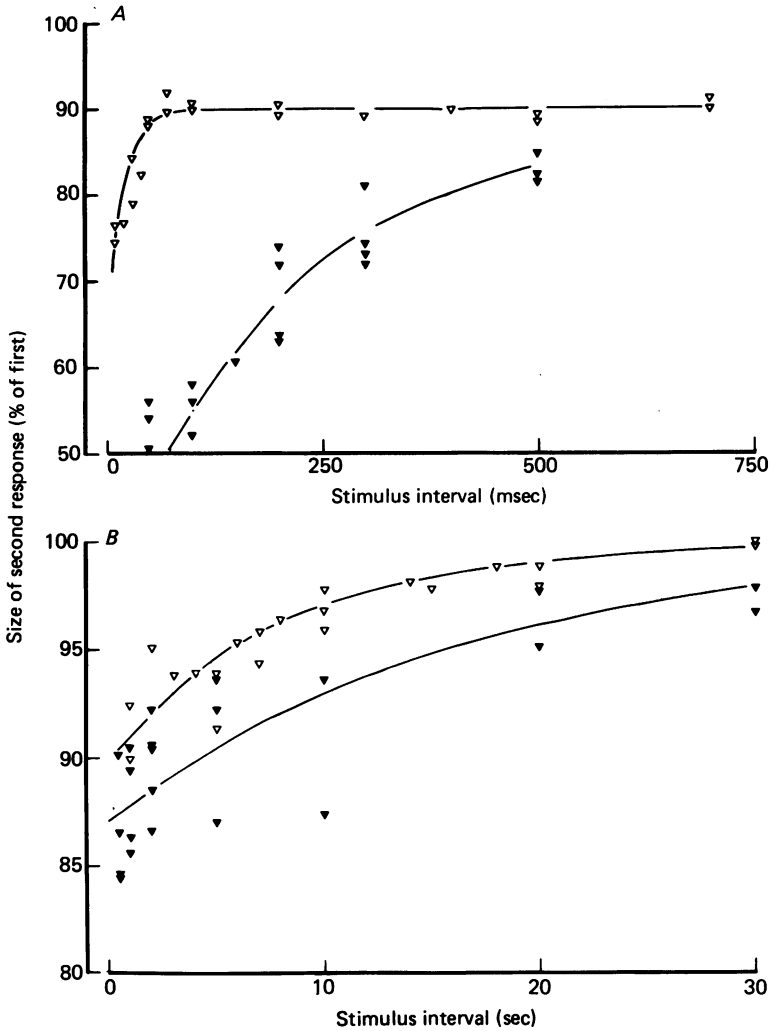

Fig. 3. Time course of recovery of fast (*A*), and slow (*B*) components, measured with paired action potentials at different temperatures. Measurements were made in the same way as for Fig. 2, and each point is a single value. Points marked ▽ were obtained from four fibres at a temperature of 25 °C, and points marked ▼ from four fibres at 6 °C. Curves fitted to the data are exponentials with time constants of: 25 °C, τ_{fast} = 20 msec; τ_{slow} = 8 sec; 6 °C, τ_{fast} = 180 msec; τ_{slow} = 16 sec.

a stimulus interval of 20 msec, the second response is only about 30 % of the first, but has increased to about 90 % at an interval of 300 msec (Fig. 2*A*). The eventual recovery of the remaining 10 % is however still not quite complete after 30 sec (Fig. 2*B*). These two components of the recovery can be fitted well by exponential functions, with time constants of 77 msec and 11 sec for the data shown.

Temperature dependence of the recovery time course

Experiments similar to that in Fig. 2 were carried out at different temperatures to examine the temperature dependence of the two components of recovery of the

arsenazo response, measured with paired action potentials. Examples of results at the two extreme temperatures used (6 and 25 °C) are shown in Fig. 3. At both temperatures the recovery time course is characterized by distinct fast and slow components. The effect of temperature on the two components is however clearly different. Increasing temperature from 6 to 25 °C speeds up the fast recovery component by a factor of about nine (time constants respectively 180 and 20 msec), but speeds the slow component by a factor of only about two (time constants 16 and 8 sec).

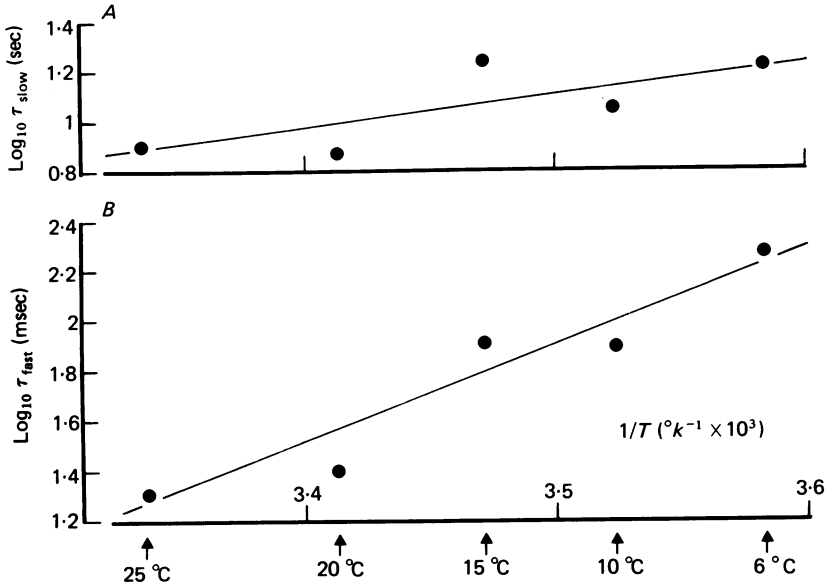

Fig. 4. Arrhenius plots showing the temperature dependence of the slow (*A*) and fast (*B*) recovery components recorded with paired action potentials. Each point is the time constant of the exponential fitted to data from at least four fibres, as in Figs. 2 and 3. Regression lines are fitted to the data.

The temperature dependence of the fast and slow recovery time constants are shown on Arrhenius plots in Fig. 4. Both components can be fitted adequately by straight lines on these plots, but the slope is much steeper for the fast component. The fast component has an approximate Q_{10} of 2·7 between 10 and 20 °C, whilst the corresponding Q_{10} for the slow component is 1·4.

Arsenazo response recovery in Ringer solution containing no added Ca^{2+}

Recovery of the arsenazo signal using paired action potentials was measured from four fibres bathed in a Ringer solution containing no added Ca^{2+}, and with 5 mM-$MgCl_2$ and 1 mM-EGTA. The results are shown in Fig. 5. The recovery time course shows two exponential components, as in normal Ringer solution, with time constants of 98 msec and 32 sec. Corresponding time constants in normal Ringer solution are 77 msec and 11 sec (measurements from different muscles). 0 Ca^{2+} solution therefore appears to produce a marked lengthening of the slow recovery component, but has little effect on the fast component.

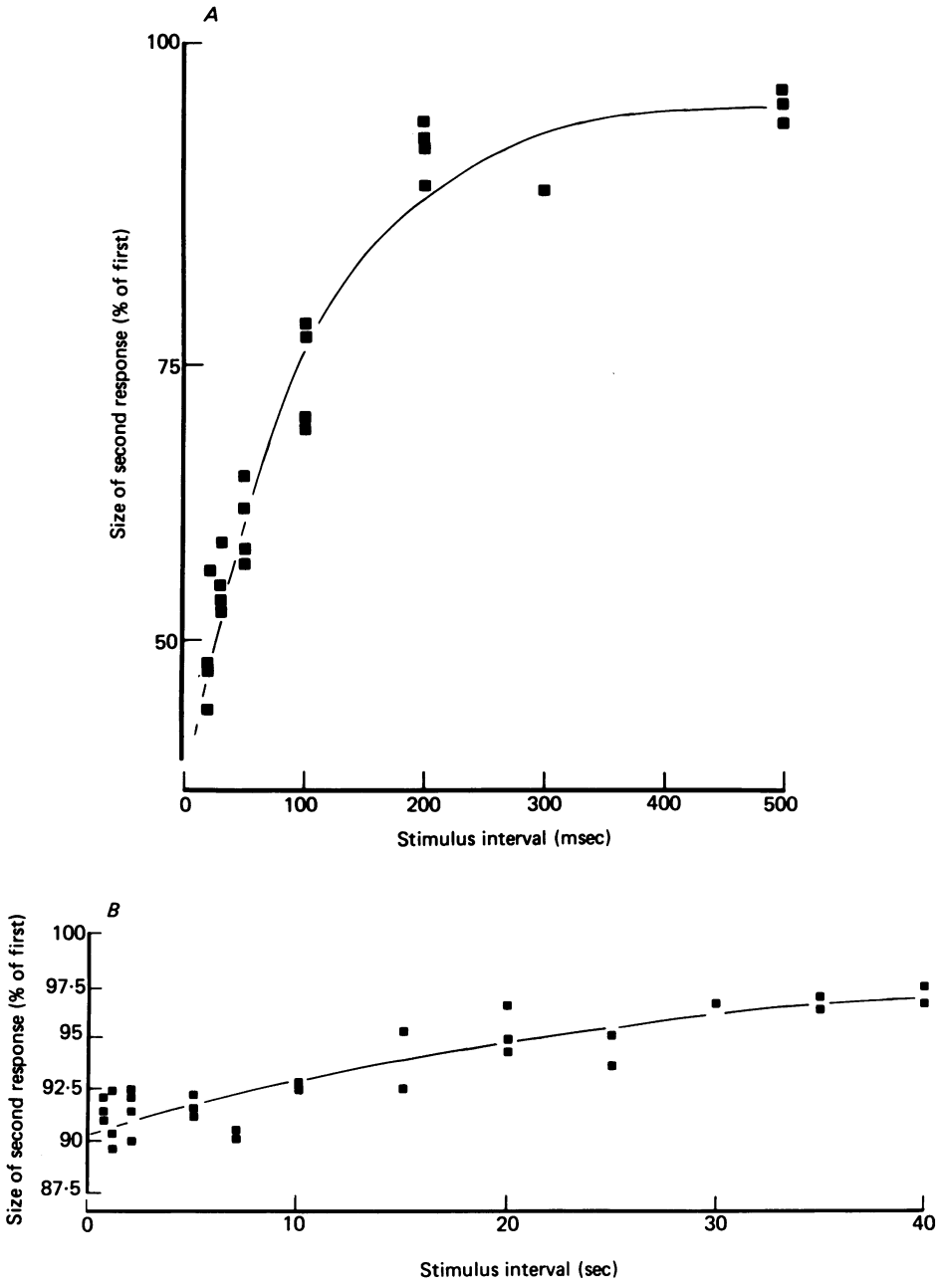

Fig. 5. Time courses of fast (*A*) and slow (*B*) recovery components measured with paired action potentials in 0 Ca^{2+} Ringer solution. Measurements were obtained from four fibres at 9–11 °C, in the same way as Fig. 2. Because of the slow recovery rate in this solution, an interval of 120 sec was allowed between tests. Exponentials fitted to the data have time constants of: τ_{fast} = 98 msec, τ_{slow} = 32 sec.

The 0 Ca^{2+} solution contained 5 mM-Mg^{2+}, in order to maintain the resting potential of the fibres. To test whether the effect of this solution was due to the absence of Ca^{2+}, or the addition of Mg^{2+}, we performed control experiments where 5 mM-Mg^{2+} was added to the normal Ringer solution. No significant changes were seen with addition of Mg^{2+}; the slow recovery time constant was 15 sec measured from three fibres in normal Ringer solution, and 16 sec measured from three other fibres from the same muscle after addition of Mg^{2+}.

In contrast, Sr^{2+} ions and Ni^{2+} ions are able to prevent the slowing of the late recovery component seen in 0 Ca^{2+} solution. For example, in one experiment a mean slow recovery time constant of 36 sec was obtained from four fibres in 0 Ca^{2+} solution (including 5 mM-Mg^{2+} and 1 mM-EGTA), whilst after changing to a Ringer solution where 2·5 mM-$SrCl_2$ was substituted for $CaCl_2$ the mean value from four other fibres was 14·5 sec. Similarly, in another experiment a value of $\tau_{slow} = 20$ sec was obtained from four fibres in 0 Ca^{2+} solution including 2·5 mM-Ni^{2+}, as compared to a control value of 23 sec from three other fibres after returning to normal Ringer solution.

Voltage dependence of response recovery

The time course of recovery of the arsenazo signal was investigated with the membrane potential held to different levels between stimuli. For these experiments we used stimulation by voltage-clamped depolarizing pulses, rather than action potentials, since large changes in action-potential duration and overshoot were observed when the fibre membrane potential was shifted by current injection. An additional KCl-filled micro-electrode was used as the current-passing electrode for the voltage clamp. The measuring light slit (about 200 μm in length) was positioned to include the tips of both recording and current-passing electrodes. Thus, optical measurements were made from a restricted length of the fibre, which would have been under good voltage-clamp control.

Stimuli were 5 msec duration pulses to about +20 mV, and the holding potential was set to either low (−60 or −80 mV) or high (−110 or −140 mV) potentials before and during each pair of pulses. Recordings at both low and high holding potentials were generally obtained for each stimulus interval. The response size elicited by a given depolarizing pulse was facilitated at the less negative holding potential (Miledi *et al.* 1981), and so the amplitude of the conditioning and test pulses was reduced at this holding potential so as to give roughly equal response sizes at both potentials (Fig. 6*A*). Muscles were treated with tetrodotoxin (2×10^{-7} g ml^{-1}) (TTX) and tetraethylammonium bromide (TEA) (20 mM) to improve clamp performance, by blocking voltage-activated Na^+ and K^+ conductances.

At short stimulus intervals (< 300 msec) there is no clear difference between the recovery time courses at holding potentials of −60 or −110 mV. Both sets of data can be fitted by an exponential with a time constant of 92 msec (Fig. 6*B*). This value is similar to that observed with action potentials at the same temperature.

Measurements at long stimulus intervals were difficult, because changes in the holding potential often caused slight changes in amplitude of the conditioning and test pulses. Fig. 6*C* shows results from one experiment where the voltage-clamp control was particularly good. There are no significant differences between the slow recovery time courses at the holding potentials at −80 or −140 mV. Both sets of data fit well to an exponential with a time constant of 16 sec.

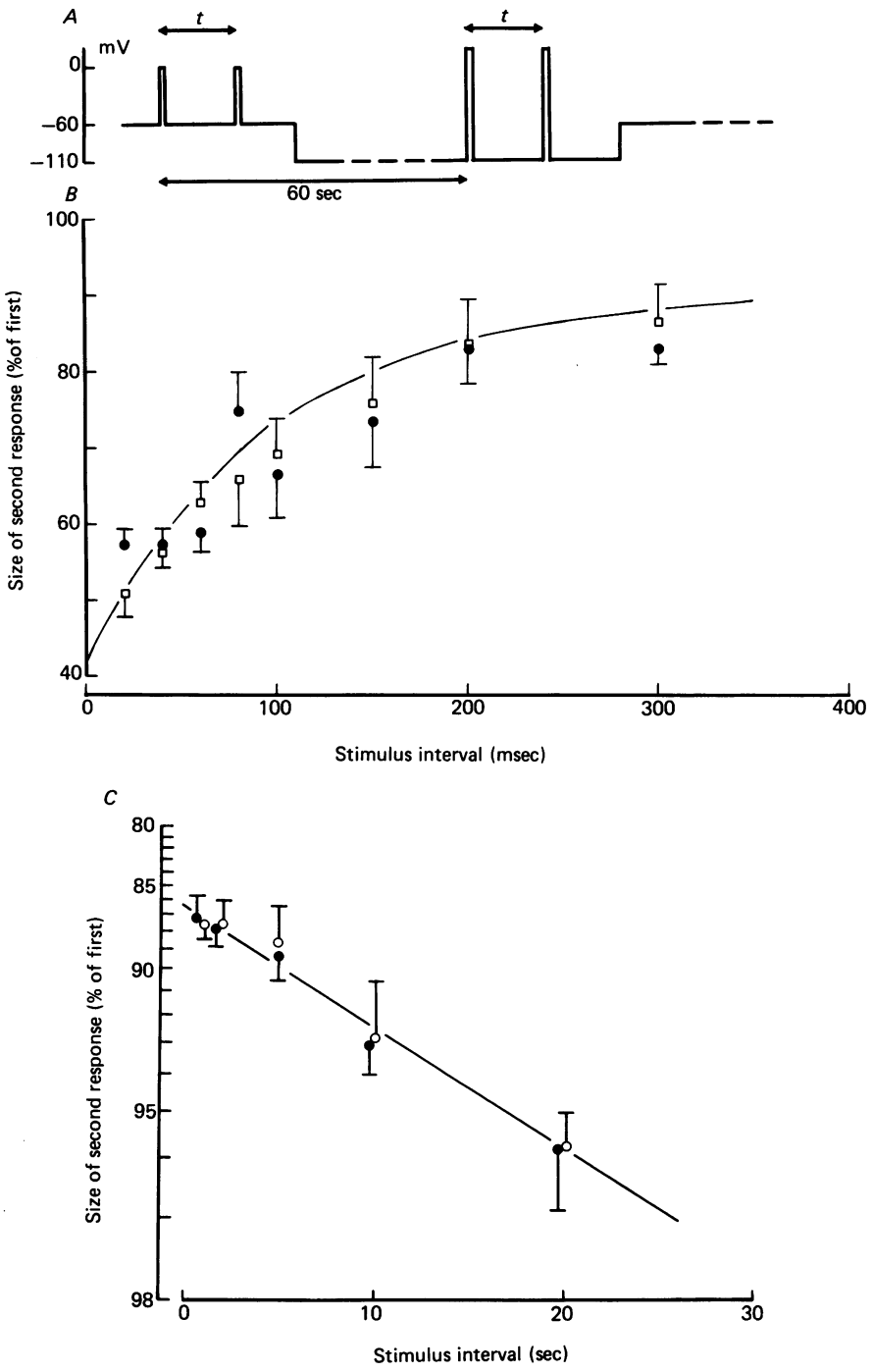

Fig. 6. Fast and slow components of arsenazo response recovery measured with paired depolarizing pulses at different holding potentials. *A*, pulse protocol. Stimulus interval is *t* and test pulse duration 5 msec. See text for details. *B*, recovery of arsenazo response amplitude at short stimulus intervals, plotted in the same way as Fig. 2*A*. Points marked

Recovery of arsenazo response after tetanic stimulation

During repetitive stimulation the size of the calcium transient from each action potential becomes strongly reduced, and test responses elicited after a tetanus also show a reduction (Miledi *et al.* 1982; Blinks *et al.* 1978). We made a quantitative examination of the recovery of the arsenazo twitch response size following tetanic stimulation, using a procedure similar to that employed for studying recovery

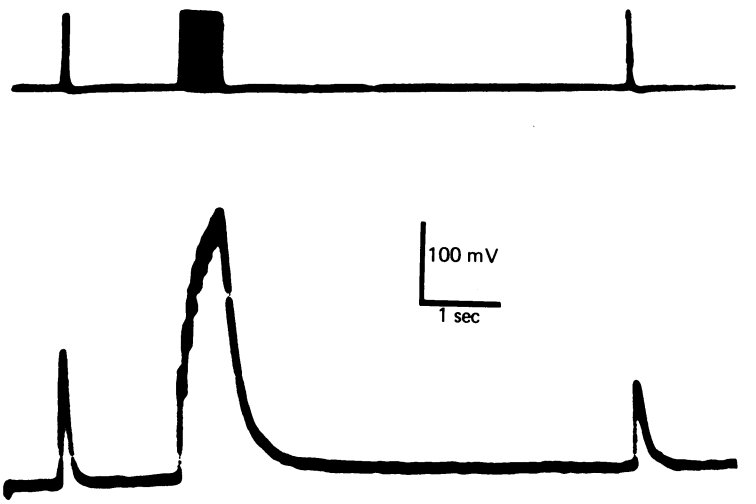

Fig. 7. Experimental protocol used to investigate the recovery of the arsenazo response to a test action potential given at various intervals following a conditioning tetanus. Upper trace shows membrane potential, and lower trace light transmission at 650–700 nm. A control action potential was elicited near the start of the sweep, followed by a conditioning tetanus (0·5 sec, 20 Hz in this example). This was then followed by a test action potential at various intervals (5 sec in this case). The size of the test response was measured as a percentage of the size of the control response. ΔI calibration, 0·01. Temperature, 9 °C.

following single action potentials. The experimental protocol is illustrated in Fig. 7. At the beginning of each record a control action potential response was recorded, and a conditioning tetanus was given 1·5 sec later. Test action potentials were then elicited at various intervals after the end of the tetanus. Only one test response was obtained in each record, and an interval of 90 sec was allowed between records. The size of the test arsenazo response is expressed as a percentage of the control response at the beginning of each record.

In the example of Fig. 7 it can be seen that the size of the arsenazo response is

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□ are data obtained at a potential of −110 mV, and points marked ● at −60 mV. Error bars are ±1 S.E. Data from seven fibres at 9–11 °C. The fitted exponential has a time constant of 92 msec, and an asymptote at 90 %. *C*, recovery of response at long stimulus intervals, plotted on semilogarithmic co-ordinates. Holding potential was −140 mV for points marked ○, and −80 mV for points marked ●. The line fitted to the data corresponds to an exponential with a time constant of 16 sec. Data are from four fibres (different to *B*) observed at both potentials, and from two additional fibres at −80 mV. Temperature, 9–11 °C.

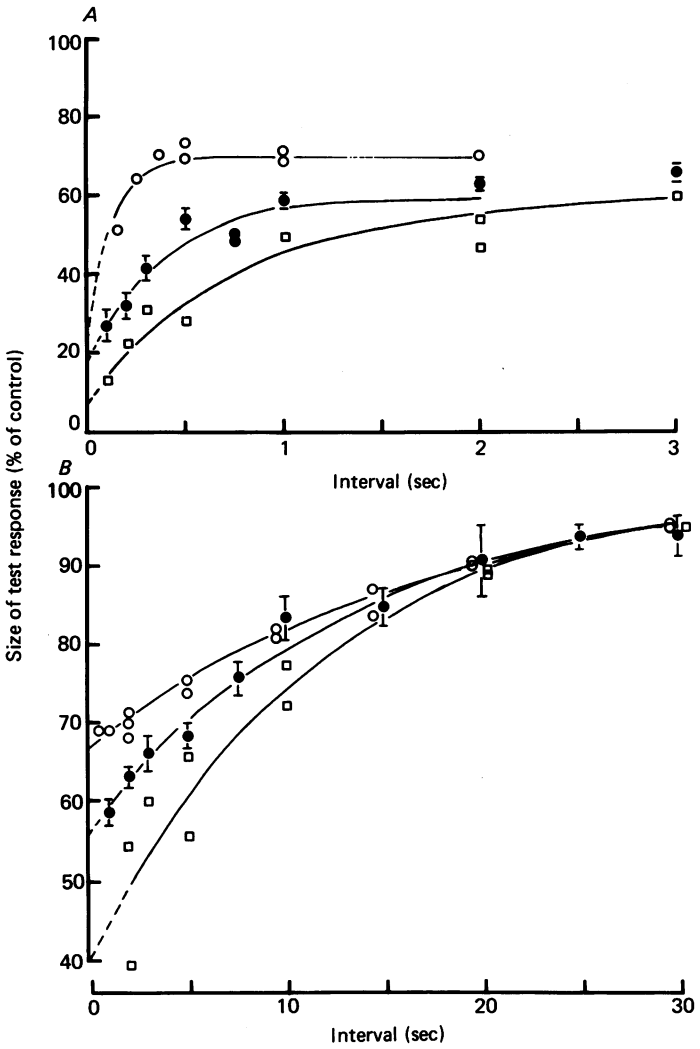

Fig. 8. Time course of fast (*A*) and slow (*B*) recovery of the test response following conditioning tetani. Data are plotted in the same way as Fig. 2. Results are shown for three different conditioning tetani; 0·2 sec, 20 Hz (○); 0·5 sec, 20 Hz (●); and 2 sec, 30 Hz (□). Error bars are ±1 S.E., points without bars are single measurements. Four fibres were examined using the 0·5 sec 20 Hz tetanus, and two fibres for each of the other two tetani. Curves fitted to the data are exponentials with time constants in *A* of ○ = 140 msec, ● = 390 msec, and □ = 800 msec and in *B* of ○ = 14·5 sec, ● = 12·5 sec and □ = 11·7 sec. Temperature, 9–11 °C.

about 70% of the control at an interval of 5 sec after the tetanus. The time course of recovery of the test response following tetani of different frequencies and durations is shown in Fig. 8. Similar to the recovery following a single impulse, the recovery time course after tetanic stimulation follows two exponential components. The time constant of the fast component is longer than following a single action potential, and depends upon the frequency and duration of the conditioning tetanus. With a

conditioning tetanus of 20 Hz for 0·5 sec the time constant was 140 msec, whilst this increased to 800 msec following a 30 Hz tetanus for 2 sec. On the other hand, the time constant of the slow exponential component is very similar to that observed after single stimuli. Time constants of between 11·7 and 14·5 sec were measured for the data

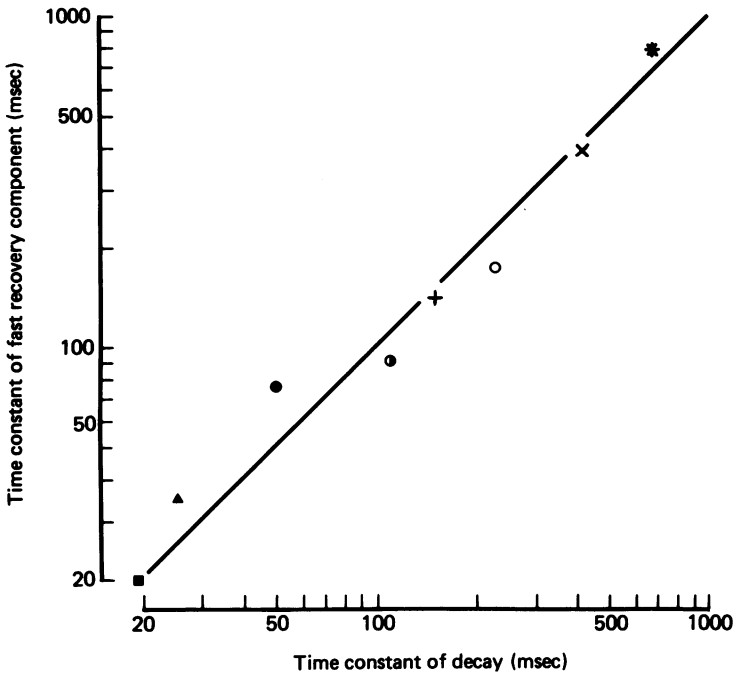

Fig. 9. Relationship between the time constant of decay of the arsenazo signal and the fast recovery time constant, measured under various conditions. The data are plotted on log–log co-ordinates because of the wide spread in values, and the line indicates a one-to-one relationship. The abscissa gives the time constant of the exponential fitted to the fast recovery component (as in Figs. 2*A*, 3*A* and 8*A*). The ordinate gives the mean decay time constant of the arsenazo response following conditioning impulses or tetani, measured from the same fibres as used to estimate the recovery time constant. Points marked ○, ◑, ●, ▲ and ■ were obtained using a single action potential as the conditioning stimulus, at temperatures of respectively 6, 10, 15, 20 and 25 °C. Conditioning tetani were used to obtain the other data points at a temperature of 9–11 °C. Stimulus parameters were: +, 0·2 sec, 20 Hz; ×, 0·5 sec, 20 Hz; *, 2 sec, 30 Hz.

in Fig. 8, and there appears to be no correlation with the frequency or duration of the tetanus. A shift is however apparent in the y-axis intercept of the extrapolated exponentials at $t = 0$ with different tetani, and at any given interval the depression of the test response due to the slow component is greater following a more intense conditioning tetanus.

Factors determining the fast recovery component

A striking observation is that the time constant of the fast exponential component of recovery corresponds closely to the decay time constant of the arsenazo response. For example, at 10 °C the decay time constant of the arsenazo signal to an action

potential is 71 msec (Miledi *et al.* 1982), whilst the time constant of the fast recovery component is 77 msec. Factors, such as temperature or tetanic stimulation, which alter the decay time constant also affect the fast recovery time constant to a similar degree. The temperature dependence of the decay time has a Q_{10} of about 2·4 (Miledi *et al.* 1982) and the temperature dependence of the fast recovery component is about 2·7. Similarly, the decay of the arsenazo signal following tetanic stimuli of increasing frequency or duration becomes progressively slower, and this is matched by a slowing in the fast recovery component following conditioning tetani.

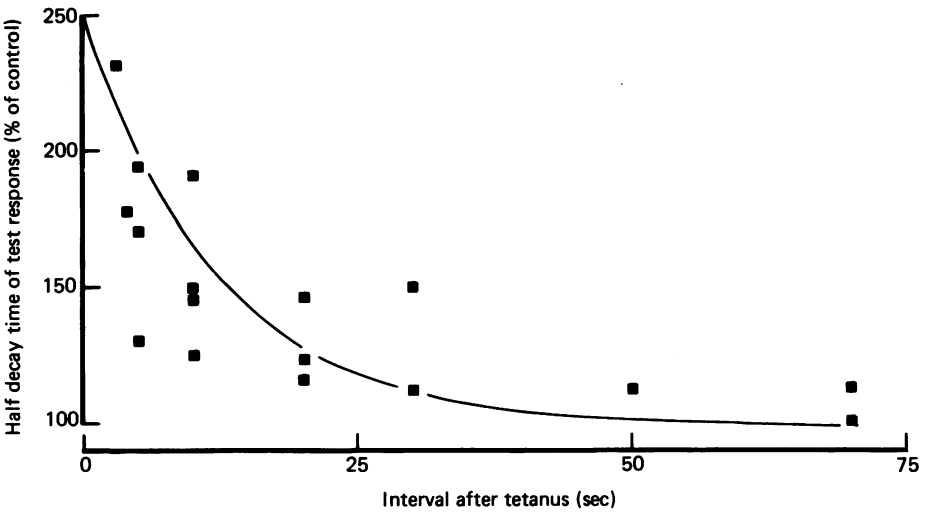

Fig. 10. Decay time constant of the arsenazo response to a test action potential at different intervals following a conditioning tetanus (20 Hz, 0·5 sec). Experimental protocol was the same as Fig. 7, and an interval of 90 sec was allowed between measurements. Responses to the control and test action potentials were recorded at fast sweep speed to allow more accurate measurement of decay times. The half decay times of the test responses are expressed as a percentage of the corresponding value for the control response elicited before the tetanus. Data are shown from four fibres, at a temperature of 9–11 °C.

The relationship between the decay time constant of the arsenazo signal and the fast recovery component is shown in Fig. 9. Decay times in this Figure were measured from the decay of the conditioning responses (single impulses or tetani) which were used to obtain the corresponding recovery times. Data are shown for both conditioning responses elicited by single action potentials, and for different conditioning tetani at 10 °C. Over a wide range of time constants, the fast recovery time follows very closely the decay time. There appear to be no systematic deviations from a one-to-one relationship over a range from 20 msec (single action potential at 25 °C) to 800 msec (2 sec; 30 Hz tetanus at 10 °C).

Decay time of arsenazo response following conditioning stimuli

The decay time of the arsenazo 'twitch' response elicited shortly after a preceding twitch or tetanus is slower than the rested-state value. This effect is quite small following a single action potential, and the half decay time of the second response

to a pair of action potentials is about 20 % slower than the first, at a stimulus interval of a few hundred milliseconds. However, the effect is more pronounced following a tetanus (Fig. 7), and we therefore investigated the changes in decay time of a test action potential signal elicited at different times after a conditioning tetanus (Fig. 10). Shortly after the tetanus (20 Hz for 0·5 sec) the half decay time of the test response is more than twice the rested-state control value, and subsequent recovery occurs over a time course of several seconds. The data in Fig. 10 have been fitted by an exponential with a time constant of 12 sec, but because of the large scatter this value is only approximate.

DISCUSSION

This paper is concerned principally with the depression and subsequent recovery in size of the Ca^{2+} transient elicited by an action potential in muscle fibres, following a conditioning stimulation. The main findings can be summarized as follows: the time course of recovery comprises two exponential components, with time constants (at 10 °C) of roughly 100 msec (τ_{fast}), and 10 sec (τ_{slow}) following a conditioning action potential. Changes in temperature have a much more pronounced effect on the time constant of the fast component than on the slow, but changes in external [Ca^{2+}] affect only the slow component. Neither component appears to be affected by changes in membrane potential. Following conditioning tetani, τ_{fast} is longer than after a single action potential, and increases with increasing duration or frequency of tetanic stimulation, whilst τ_{slow} is little changed. Under all conditions observed, τ_{fast} closely follows the time constant of decay of the arsenazo signal to the conditioning stimulus.

None of these effects appear to be due to changes in action potential size or duration. In the case of the fast component, any observed changes in action potential parameters were less than about 5 %, which is much too small to account for the 50 % or more reduction in size of the arsenazo signal. The reduction in arsenazo signal due to the slow component is smaller ($\sim$ 10 %) so for this case the argument is less certain, especially considering that the surface action potential may not accurately reflect the potential changes in the T-tubules. However, the finding that both fast and slow components remain virtually unchanged when studied using voltage-clamp pulses in TTX- and TEA-treated fibres gives a strong argument that changes in parameters of the tubular action potential play no important role. Also, measurements of action potential parameters indicated that at a 2 sec stimulus interval, the second action potential was reduced in size by about 0·5 mV, but that the duration measured at 0 mV was increased by about 3 %. On balance, a very slight increase in arsenazo response might be expected from the change in action potential.

Furthermore, it is unlikely that the observed recovery components are distorted to any appreciable extent by the properties of the arsenazo recording system. Two possibilities may be considered. First, that the arsenazo might become saturated by the initial stimulus of a pair, thus giving an apparently smaller response to the subsequent stimulus. A strong argument against this is given by the observation that arsenazo signals nearly four times larger than that elicited by an action potential can be obtained using voltage clamped depolarizing pulses. For example, a mean response size of $\Delta A/A_{570} = 0{\cdot}31$ (S.D. = 0·03, five fibres) was obtained for 20 msec depolari-

zations to +20 mV, as compared to $\Delta A/A_{570} = 0{\cdot}08$ for the action potential (Miledi *et al.* 1982). A second possible source of error is that acidification of the muscle cytoplasm following stimulation could reduce the sensitivity of arsenazo to Ca^{2+}, thus apparently reducing the size of the response to a second stimulus. However, only very small changes in pH are seen following action potentials in highly stretched fibres (Baylor, Chandler & Marshall, 1982).

Our results are in general agreement with a previous study (Rall, 1980), when the recovery of activation heat was measured in highly stretched muscles, using paired action potentials or conditioning tetani. The activation heat was presumed to give a measure of the Ca^{2+} released by each stimulus, and at a temperature of 0 °C the recovery of activation heat measured using paired action potentials followed a single exponential time course (excepting very brief intervals) with a half time of 500 msec. Following a conditioning tetanus (3 sec duration) the recovery followed two time courses, with half times of 730 msec and 34 sec (at 0 °C). Bearing in mind the difference in temperature, these time courses correspond reasonably well with our data on the recovery of the Ca^{2+} transient. The absence of a slow recovery component with paired action potentials in Rall's data may be attributable to differences in resolution of the arsenazo- and heat-measurement techniques. Also, he did not examine stimulus intervals longer than 2·5 sec.

The observation that the recovery time course of the Ca^{2+} transient follows two exponential components with very different time constants suggests that two separate mechanisms may be involved. This view is strengthened by the observation that the Q_{10} of the fast component is much greater than the slow component ($Q_{10} = 2{\cdot}7$ and 1·4 respectively).

Fast recovery component

A strong clue about the origin of the fast component is provided by the finding that τ_{fast} appears always to closely follow the decay time constant of the arsenazo response elicited by the conditioning stimulus. This one-to-one relationship holds over at least a forty-fold range of time constants, and for two different parameters which alter the time constants (temperature, and number of impulses in the conditioning stimulus). Since the decay phase of the arsenazo signal probably reflects the uptake of Ca^{2+} ions by the s.r. (Miledi *et al.* 1982), then a simple explanation for the fast recovery component is that a part of the reduction in size of the Ca^{2+} transient following conditioning stimuli results from depletion of Ca^{2+} stores available for release in the s.r. Subsequent recovery of the response size would then occur due to re-filling of these stores by Ca^{2+} ions which are taken up by the s.r. from the cytoplasm.

This explanation is attractive, but there are other possible mechanisms which could account for the observed correspondence between the time constants of the fast recovery component and the response decay. For example, the amount of Ca^{2+} released by a following test stimulus might be depressed because of the elevated cytoplasmic free Ca^{2+} concentration following a conditioning action potential or tetanus. Estimates of the total amount of Ca^{2+} released from the s.r., however, indicate that during a tetanus the majority of the fibre Ca^{2+} will be re-distributed from the s.r. to the cytoplasm (Miledi *et al.* 1982; Somlyo, Gonzalez-Serratos, Shuman, McClellan & Somlyo, 1981). Thus, depletion and re-filling of Ca^{2+} stores in the s.r.

is expected to play a large role in determining the recovery of the Ca^{2+} transient size, even if other mechanisms may also be involved.

This model involving depletion and rapid re-filling of the s.r. by Ca^{2+} is at variance with a previous hypothesis as proposed by Winegrad and colleagues (Winegrad, 1968, 1970; Connolly *et al.* 1971), where the rate-limiting step in recovery is presumed to result from the translocation of Ca^{2+} ions from the longitudinal s.r. to release sites in the terminal cisternae. Movement of Ca^{2+} ions in these experiments was followed using labelled $^{45}Ca^{2+}$ ions, and suggested that movement of Ca^{2+} within the s.r. has a half time of about 28 sec at 10 °C following a long tetanus. If this observation is correct, then our fast recovery component would have to arise from some process affecting Ca^{2+} release from the terminal cisternae, and the correspondence with the rate of uptake of Ca^{2+} from the myoplasm would be merely coincidental.

Recent measurements using electron-probe analysis (Somlyo *et al.* 1981) have, however, thrown doubt on the interpretation of Winegrad and collaborators. The higher resolution available with the electron probe suggests that the labelled Ca^{2+}, which was interpreted as being within the longitudinal s.r., is in fact bound within the cytoplasm. No appreciable changes in Ca^{2+} concentration of the longitudinal s.r. were seen following tetanic stimulation (Somlyo *et al.* 1981). Some additional observations of intracellular Ca^{2+} transients also argue against the existence of slow translation of Ca^{2+} ions within the s.r. For example, by summation of the individual transients during the first 500 msec of a tetanus at 40 Hz, it can be estimated that the total Ca^{2+} released is about six times that released during a single twitch (data from Fig. 10, Miledi *et al.* 1982). The amount of Ca^{2+} translocated from the longitudinal to the terminal s.r. during this time would be negligible on Winegrad's model, and so all of the release would have to be accounted for by pre-existing stores. However, it has been estimated that a single twitch may release around one quarter of the total Ca^{2+} present in the s.r. (Endo, 1977; Miledi *et al.* 1982). Hence, if there is not a rapid recycling of Ca^{2+} within the s.r., this tetanus would be expected to more than completely exhaust the stores of Ca^{2+} in the s.r., and yet Ca^{2+} release can continue for several seconds during stimulation at 40 Hz (Blinks *et al.* 1978; Miledi *et al.* 1982).

From a functional point of view, it would also seem strange if a rapid pump for uptake of Ca^{2+} ions from the myoplasm into the s.r. is followed by an internal translocation which is nearly two orders of magnitude slower. This is especially so, considering that uptake is against a large concentration gradient, and hence metabolically expensive, whilst translocation of Ca^{2+} ions along the s.r. might be expected to occur on a millisecond time scale by diffusion alone (Endo, 1977).

Slow recovery component

Our results provide less information about the origin of the slow recovery component. The only experimental procedure found which appreciably altered the time constant of this component was removal of Ca^{2+} ions from the bathing medium, an effect which could be reversed by substitution with Sr^{2+} or Ni^{2+} ions. This suggests that the slow recovery may involve some structure in the muscle which is accessible to the external solution, and a likely candidate is the membrane of the T-tubules, which is involved in the e–c coupling mechanism. It has been proposed that this

mechanism may become inactivated during depolarization (Frankenhaeuser & Lännergren, 1967; Caputo, 1972, 1981; Caputo & DeBolaños, 1979) and alterations in external Ca^{2+} level appear to affect the rate of inactivation (Miledi, Parker & Schalow, 1979, 1981). Charge movement recorded from muscle fibres shows a similar inactivation and it has been suggested that it may reflect the movement of gating particles involved in e–c coupling (Chandler *et al.* 1976; Adrian *et al.* 1976; Rakowski, 1976; Kovács *et al.* 1979; Schneider, 1981). Recovery from inactivation following prolonged depolarizations (repriming) has been examined using contractile responses and charge movement (Adrian *et al.* 1976), but the repriming process measured after prolonged depolarizations shows markedly different properties to our slow recovery component. Notably, the rate of repriming has a very high temperature dependence (Q_{10} of 9) and is faster at more negative holding potentials. In contrast the slow recovery component has a Q_{10} of 1·4 and shows no appreciable voltage dependence. Differences in experimental procedures (particularly the conditioning stimuli) however make comparison of the results uncertain, and a full analysis must await simultaneous measurements of charge movement and recovery of the Ca^{2+} transient.

Although we favour the idea that the slow recovery component arises from inactivation of the e–c coupling process, other mechanisms are possible. One of them is that part of the Ca^{2+} released from the s.r. may be sequestered by some compartment in the muscle (e.g. mitochondria, T-tubule lumen, binding to parvalbumin), from which it is released over a time course of several seconds and taken up again by the s.r. The extent of the reduction in Ca^{2+} transient due to the slow recovery component following prolonged tetani indicates that on this model over one half of the released Ca^{2+} would have to be sequestered in this way. Electron-probe measurements (Somlyo *et al.* 1981) indicate that there is little change in mitochondrial [Ca^{2+}] during tetani, but that appreciable amounts of Ca^{2+} may be bound to parvalbumin. In resting fibres, the binding sites on parvalbumin are occupied by Mg^{2+} ions, and because the rate of Mg^{2+} removal is slow (half time $\sim$ 0·5 sec; Potter, Robertson & Johnson, 1982) it is expected that much more Ca^{2+} binding to parvalbumin will occur during tetani than during a twitch. The characteristics of the slow recovery component could thus be well accounted for by Ca^{2+} binding to parvalbumin, with the only exception being that it is difficult to see how the rate of Ca^{2+} dissociation from parvalbumin might depend on the divalent cation composition of the bathing solution.

Ca^{2+} transients during tetani

The data presented here form a basis for understanding the Ca^{2+} transients observed during tetanic stimulation of muscle (Blinks *et al.* 1978; Miledi *et al.* 1982), although in view of the complexity of the factors involved we have not attempted to quantitatively model the responses.

The myoplasmic free Ca^{2+} level during a tetanus will be influenced by three factors which we have considered: (i), reduction in the amount of Ca^{2+} released by successive impulses due to the process underlying the fast recovery component; (ii), reduction in Ca^{2+} release arising from the slow recovery component, and (iii), progressive slowing of decline of free myoplasmic Ca^{2+} following increasing number of stimuli. During tetani of moderate duration, factors (i) and (iii) are likely to be the most

important. For example, at the end of a 2 sec tetanus at 30 Hz, a reduction in Ca^{2+} release by a factor of about six is observed due to the fast recovery component (data from Fig. 8*A*). The rate of decline of myoplasmic free Ca^{2+} is also slowed by a factor of about six (Fig. 9). The observed decline in Ca^{2+} release due to the slow recovery component on the other hand, is by a factor of only about two (Fig. 8*B*). Since the time constant of the fast recovery component is slowed in parallel with the lengthening in uptake time constant during a tetanus, the net effect will be such that a given myoplasmic Ca^{2+} level can be maintained with progressively less cycling via s.r. uptake and release.

With increasing durations of tetanic stimulation the extent of depression of Ca^{2+} release due to the slow component increases, and would eventually be expected to cause the myoplasmic Ca^{2+} level to fall during a long tetanus. This effect may not be important under physiological conditions of stimulation, since we generally find that the action potential fails during prolonged stimulation before there is any appreciable decline in the arsenazo signal. However, during prolonged depolarization of a fibre by K^+, or by a voltage-clamped pulse, the myoplasmic Ca^{2+} level does fall during stimulation (Blinks *et al.* 1978; Miledi *et al.* 1982), and this is probably due to depression of release arising from the slow recovery component.

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