

CALCIUM TRANSIENTS IN NORMAL AND DENERVATED SLOW MUSCLE FIBRES OF THE FROG

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SUMMARY

1. Intracellular changes in free Ca^{2+} concentration were recorded from slow muscle fibres in the pyriformis muscle of *Rana temporaria*, using the dye arsenazo III. Fibres were voltage clamped, and arsenazo signals were recorded in response to depolarizing pulses.

2. The size of the arsenazo response to depolarizing pulses of 100 msec duration was a sigmoid function of membrane potential over the range -45 to 0 mV, and remained constant with further depolarizations up to $+100$ mV.

3. The peak size of the arsenazo signal to supramaximal depolarizations increased with increasing pulse length. The initial rising phase during a pulse was much slower than in twitch fibres, and this phase was followed by an even slower rise. Following short pulses the decay of the response was exponential, with a time constant of about 1.4 sec, while after long pulses the decline became much slower.

4. Decreasing free Ca^{2+} concentration in the bathing medium to very low levels, using EGTA, did not affect the responses to short (100 msec) depolarizations.

5. Slow fibres bathed in Ringer's solution containing 12 mM- Ca^{2+} showed a well maintained arsenazo response to supramaximal depolarizations lasting over 1 min. Reduction of external Ca^{2+} to 1.8 and (nominally) 0 mM caused the response to become progressively more transient.

6. After denervation, slow fibres developed action potentials, but none of the parameters of the arsenazo response was significantly changed. During the early phase of reinnervation by a mixed nerve, when fast conducting axons begin to innervate slow fibres, the ability to give a maintained response during long depolarizations was reduced.

7. It is concluded that intracellular Ca^{2+} transients in slow muscle fibres are probably generated by a similar mechanism as in twitch fibres and entry of external Ca^{2+} is not an appreciable factor. The slow time course of the transients may be important in determining the time courses of tension development and relaxation.

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INTRODUCTION

Many muscles in the frog possess two clearly distinct types of fibres, twitch and slow fibres, which differ in several respects (see reviews by Peachey, 1968; Hess, 1970; Costantin, 1975*a,b*; Lännergren, 1975). Slow fibres give graded slow contractions with nerve stimulation, and unlike twitch fibres can maintain a contracture during prolonged depolarization. It is now thought that contractile activation in slow fibres is mediated by a rise in myoplasmic free Ca^{2+} released from internal stores in a manner similar to that in twitch fibres (Costantin, Podolsky & Tice, 1967; Flintney, 1971; Franzini-Armstrong, 1973; Miledi, Parker & Schalow, 1977*a, b*; Gilly & Hui, 1980), although the role of external Ca^{2+} during maintained contractures remains unclear (Lännergren, 1967*a*; Miledi *et al.* 1977*b*; Gilly & Hui, 1980).

Slow fibres do not normally produce action potentials in response to either direct or nerve stimulation (Kuffler & Vaughan Williams, 1953, Burke & Ginsborg, 1956). Both the electrical and mechanical properties of the fibres are however neurally controlled, and after denervation slow muscle fibres acquire the action potential mechanism (Miledi, Stefani & Steinbach, 1971). The ability to produce a maintained contracture remains in denervated fibres, but is lost after reinnervation by fast axons (Miledi & Orkand, 1966) or by a mixed nerve (Elul, Miledi & Stefani, 1970; Lehman & Schmidt, 1979), probably because reinnervation is initially made by the fast conducting axons which normally innervate twitch muscle fibres (Elul *et al.* 1970; Schmidt & Stefani, 1976, 1977). This phase is transient; the fibres lose the action potential mechanism, and become able again to generate sustained contractures, when slowly conducting axons re-establish normal synaptic contacts (Elul *et al.* 1970; Lehmann & Schmidt, 1979).

We have used the Ca indicator dye arsenazo III (Miledi *et al.* 1977*a*; Parker, 1979) to record intracellular Ca^{2+} transients in frog slow muscle fibres, and to examine how these are related to the known electrical and mechanical events occurring in normal, denervated, and reinnervated fibres. A preliminary account of some of the work on normally innervated fibres has appeared (Miledi *et al.* 1977*b*).

METHODS

Experiments were performed on the pyriformis muscle of *R. temporaria*, at 6–9 °C. The bathing solution had the following composition (mM); NaCl, 120; KCl, 2; CaCl_2 , 12; at pH 7.2. The Ca^{2+} concentration was sometimes varied, as stated in the text. To examine the effects of denervation the nervus ischiadicus was cut, leaving a nerve stump of about 10 mm length (Miledi, 1960; Schmidt & Stefani 1976, 1977). To study reinnervation the nerve was crushed. After the operation, frogs were kept in plastic tanks at about 18 °C.

Intracellular free Ca^{2+} changes were monitored with arsenazo III as described previously (Miledi *et al.* 1977*a*; Parker, 1979). Briefly by cutting away the lower part, the muscle was reduced to a thin sheet of fibres, which was sufficiently transparent for optical recording. The muscle was then stretched until contraction was practically abolished when stimulating directly with external wire electrodes. Fibres were impaled with two micro-electrodes about 150 μm apart, for voltage recordings and dye injection. A tentative identification of slow fibres was made visually, and identification was confirmed by the slow electrical time constant when tested with current pulses applied through the dye pipette. The membrane potential was held to -100 mV after penetration, and this served to inject arsenazo III into the fibre over several minutes. The dye injection pipette was filled with a 1 mM solution of arsenazo III (Sigma Chemical Co. – practical grade), which had

not been specially treated to remove contaminating Ca²⁺. During recording the region of the fibre between the two micro-electrodes was used for optical measurements, and was voltage-clamped using a conventional two point clamp with the dye pipette serving as the current electrode.

Changes in light absorbance of the injected dye were monitored by focussing light onto a spot of about 80 μm diameter on the fibre, and measuring the transmitted light at wave-lengths of 532 and 602 nm, using two photomultipliers and interference filters. When it binds Ca²⁺, the light absorbance of arsenazo changes in opposite directions at these two wave-lengths, and by subtracting the signals from the two photomultipliers a record was obtained which gave a measure of changes in free Ca²⁺ concentration, but was little affected by any movement artifacts. In order to standardize for differences in fibre diameter and amounts of arsenazo injected, the absorbance changes were expressed as a standardized change ΔA_s by dividing by the resting absorbance of the injected dye at 532 nm

$$\Delta A_s = (\Delta A_{532} - \Delta A_{602}) / A_{532}$$

This method of standardization assumes a linear relationship between light response and dye concentration in the muscle fibre. We have found this to hold true over much of the range of concentrations used in these experiments (cf. Parker, 1979), although a square-law relationship with dye concentration has also been reported (Thomas, 1979). For free Ca²⁺ levels up to about 10 μM the light absorbance signal increases linearly with free Ca²⁺ concentration (Parker, 1979; Miledi, Parker & Schalow, 1980), but begins to saturate at higher levels. Over the linear range we have found a calibration factor of 20 μM per unit ΔA_s (Miledi *et al.* 1977a), although this value should be regarded as approximate as the sensitivity is affected by several factors, including intracellular pH and Mg²⁺ concentration.

RESULTS

Normal muscles

Arsenazo responses to depolarizing pulses

Depolarization of slow fibres gave rise to slow changes in light absorbance of intracellularly injected arsenazo III. Fig. 1 shows light absorbance records obtained from an arsenazo loaded slow fibre, in response to voltage clamped depolarizing pulses of various durations. These changes resulted from a change in the absorption spectrum of the injected dye, and were not significantly contaminated by movement artifacts. Signals were generally recorded at the wave-length pair 532–602 nm, which shows a large change when arsenazo binds Ca²⁺ ions, but offers a substantial cancellation of any absorbance changes due to movement of the fibre. As a check for the presence of movement artifacts simultaneous records were also obtained at 532 nm. This trace is included in Fig. 1A and displays only the small change expected from the change in absorbance of the dye. Before dye injection no significant response was present at the difference wave-length pair.

The size of the arsenazo light response was a graded function of both pulse size and duration. The depolarizations used in Fig. 1 were of supramaximal amplitude (cf. Fig. 2), and the rates of rise and fall of the arsenazo signal were both considerably slower than previously seen in twitch muscle fibres (cf. Miledi *et al.* 1977a).

For pulse durations of about 100 msec and shorter, the decline of the response after the end of the pulse could be fitted well by an exponential (Fig. 1B). The decay time constant (time to fall to 1/e) measured in ten fibres using a 100 msec pulse was 1.4 ± 1.2 sec, but as indicated by the large standard deviation, a wide variation was seen between different fibres, with an extreme range of 0.6–4 sec. Some of this variation may be due to unavoidable damage caused by insertion of the electrodes. With longer pulses the decline of the response became slower and did not follow a

single exponential. For example, in Fig. 1 *A* the initial rates of decline were practically the same following the three longest pulses, and after depolarizations lasting about 1 min the time for the response to decline to one half could be as long as 15 sec (Figs. 4*A* and 9*C*). This slowing of the decline of the light response with long pulses is similar to that seen in twitch fibres (Miledi *et al.* 1977*a*), and suggests that the Ca^{2+} re-uptake mechanism may become saturated by long periods of high myoplasmic Ca^{2+} levels.

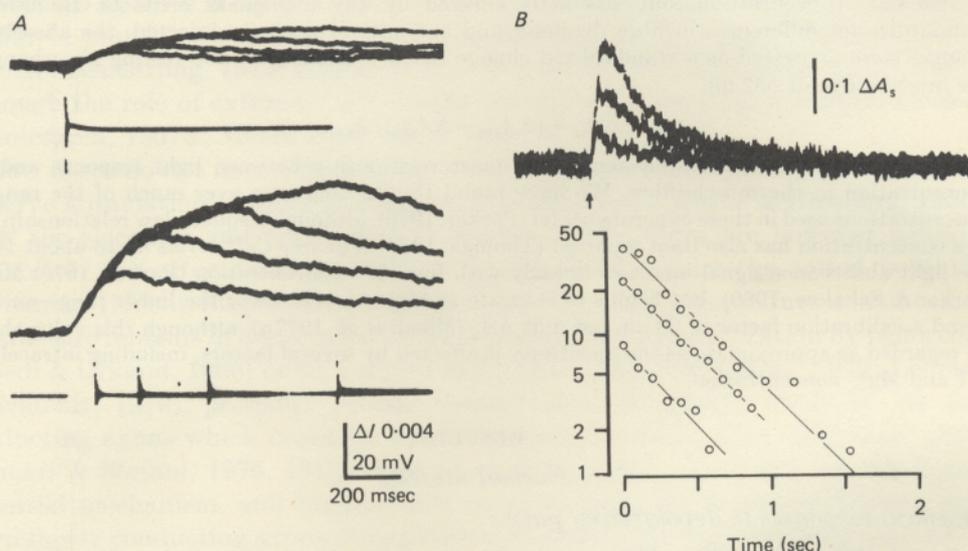


Fig. 1. Light absorbance records from two slow fibres injected with arsenazo III, showing superimposed responses to depolarizing pulses of different durations. Fibres were voltage clamped, and held at a potential of -100 mV between tests. Ringer solution contained 12 mM- Ca^{2+} . *A*, rising phase of the arsenazo signal. Lower trace is membrane potential, and middle and upper traces respectively light transmission at $532\text{--}602$ nm and at 532 nm. The calibration bar for the 532 nm trace is the fractional change in transmitted light (ΔI) at this wave-length, and for the $532\text{--}602$ nm trace is a standardized light absorbance change ΔA_s of 0.07 . Optical traces were low pass filtered with a time constant of 33 msec. Temperature 8°C . In this, and subsequent Figures, an increase in light transmission is shown as an upward deflection. *B*, falling phase of the arsenazo signal. Traces show superimposed records of light transmission at $532\text{--}602$ nm in response to voltage clamped depolarizing pulses to 0 mV, with durations of 20 , 50 , and 100 msec. Pulses began at the time indicated by the arrow. Optical time constant was 10 msec, and temperature 7.5°C . The lower graph shows semilogarithmic plots of the decay phases of the three responses. The time scale is the same as for the original traces, and the ordinate is in arbitrary units.

The rising phase of the light response during depolarizing pulses showed two distinct phases, an initial rapid increase, followed by a much slower rate of rise (Figs. 1*A* and 6). This behaviour appears to be a property of the Ca^{2+} release mechanism of the fibre, and cannot be attributed to a non-linearity of the recording system, as the transition occurs at different times and different Ca^{2+} levels for different sized pulses (Fig. 7). A similar biphasic rise has recently been observed in tension records from single slow muscle fibres (Lehman & Schmidt, 1979). The rate of rise of the initial fast phase of the arsenazo response had a mean value of $0.0022 \pm 0.0012 \Delta A_s \text{ msec}^{-1}$

in ten fibres stimulated by supramaximal depolarizing pulses. This corresponds to a rate of rise in free myoplasmic Ca²⁺ concentration of about 0.045 $\mu\text{mole msec}^{-1}$, using the calibration factor given in Methods.

Voltage dependence of the arsenazo response

The relationship between membrane potential and size of the arsenazo response was explored in voltage clamped fibres, using depolarizing pulses of 100 msec duration to various potentials, from a steady holding potential of -90 or -100 mV (Fig. 2).

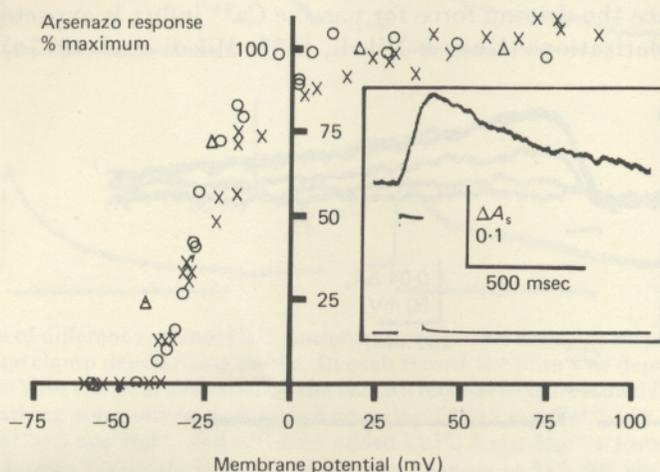


Fig. 2. Voltage dependence of the arsenazo signal elicited by 100 msec depolarizing pulses. The inset shows a typical response recorded from the same fibre as points (○) in the graph. Upper trace is light transmission at 532–602 nm, and lower trace membrane potential. Calibration bar for the potential is 50 mV, and the holding potential was -90 mV. Optical time constant was 33 msec, and temperature 7.5°C . The abscissa of the graph gives membrane potential during the pulse, and the ordinate the peak size of the arsenazo signal. Results from three fibres are shown, all in 12 mM-Ca²⁺ Ringer at a temperature of $7\text{--}8^\circ\text{C}$. To allow pooling of data from different fibres, the results have been normalized by expressing them as a percentage of the mean value at potentials above 0 mV for each fibre.

TEA (30 mM) was added to the bathing solution in these experiments to block the K⁺ conductance, and improve the performance of the clamp. The peak size of the arsenazo response was found to be a graded function of the membrane potential during the pulse, with a threshold for a detectable response at about -40 mV. At potentials above about 0 mV the response generally reached a maximum size and did not increase with further depolarization, although in one fibre examined (crosses in Fig. 2) a slight increase was observed over the range 0–30 mV.

The threshold potential for the arsenazo response is somewhat more depolarized than the value of about -55 mV reported for detection of a visible contraction in response to 100 msec depolarizing pulses (Gilly & Hui, 1980). The difference may be due to the higher concentration of Ca²⁺ in the Ringer in our experiments, resulting in a difference in screening of surface charges on the muscle membrane. A contraction threshold of -40 mV has been reported in twitch muscle fibres bathed in a solution

containing 10 mM-Ca²⁺ (Costantin, 1968), and which shifted to about -47 mV in 2mM-Ca²⁺ solution. It is also possible that the visual threshold at contraction is a more sensitive monitor than the arsenazo recording.

Role of external Ca⁺² in the generation of the Ca²⁺ transient

The peak amplitude of the arsenazo response produced by large depolarizing pulses remained essentially constant within the range 0 to +100 mV (Fig. 2). This suggests that the entry of external Ca²⁺ from the bathing solution is not important for the generation of the intracellular Ca²⁺ transient with relatively short depolarizations (100 msec), since the driving force for passive Ca²⁺ influx is expected to be reduced by larger depolarizations (Katz & Miledi, 1967; Miledi *et al.* 1977*a*).

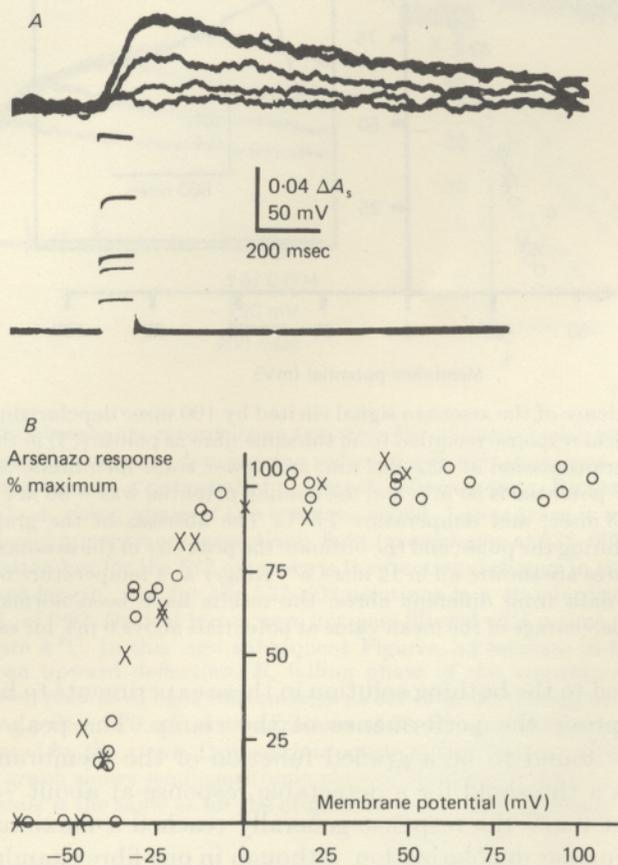


Fig. 3. Voltage dependence of the arsenazo signal in zero Ca²⁺ + EGTA solution. Depolarizing pulses of 100 msec were used, and the experiments were carried out in the same way as for Fig. 2. Muscles were rinsed several times in the zero Ca²⁺ + EGTA solution, and remained in this solution for at least 1 hr before records were obtained. *A*, superimposed arsenazo responses to 100 msec depolarizing pulses of different sizes. Lower trace is membrane potential, and upper trace light transmission at 532–602 nm. Optical time constant was 33 msec, and temperature 7 °C. The light responses to the two largest pulses superimposed. *B*, voltage dependence of the peak size of the arsenazo response. Data are shown from two fibres (different muscles), and have been normalized as in Fig. 2.

This point was further examined by bathing muscles in a Ringer solution containing no added Ca²⁺ and 1 mM-EGTA. 5 mM-MgCl₂ was also included in this solution in order to prevent the fall in resting potential seen in fibres bathed in solutions containing very low concentrations of divalent cations. The free Ca²⁺ concentration in this solution would have been less than 10⁻⁷ M, and depolarizations

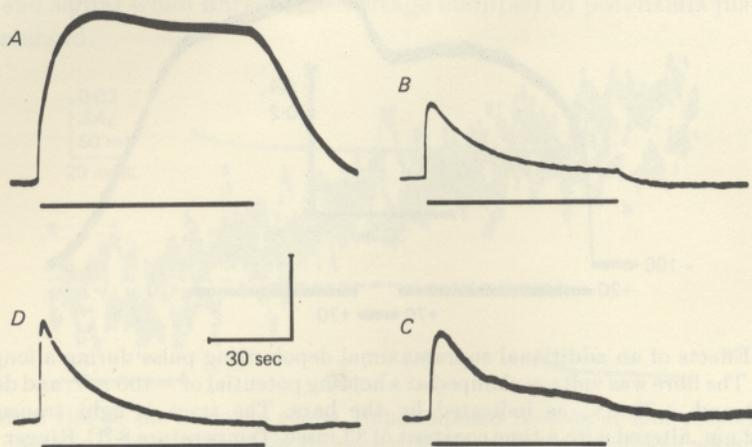


Fig. 4. Effects of different external Ca²⁺ concentrations on the arsenazo responses elicited by long voltage clamp depolarizing pulses. In each record the fibre was depolarized from -90 to $+20$ mV for the time indicated by the bar. All records were obtained from different fibres. The bathing solutions were Ringer containing (A) 12 mM-Ca²⁺, (B) 1.8 mM Ca²⁺, (C) 1.8 mM-Ca²⁺ + 5 mM-Mg²⁺, and (D) zero added Ca²⁺, 5 mM-Mg²⁺ + 1 mM-EGTA. pH was 7.2 in each case. Traces show light transmission changes at 532–602 nm, with a filter time constant of 33 msec. The calibration bar corresponds to a change in standardized light absorbance (ΔA_s) of 0.26 in (A), and 0.4 in the other records. Time calibration, 30 sec. Temperature 7 °C.

to positive membrane potentials would be expected to give rise to a reversed electrochemical gradient for Ca²⁺ movement. However, no important changes in the Ca²⁺ transients produced by large 100 msec depolarizing pulses were detected under these conditions (Fig. 3). The mean peak response size in EGTA solution was $0.07 \pm 0.02 \Delta A_s$ (four fibres), compared with $0.1 \pm 0.05 \Delta A_s$ (nine fibres) in 12 mM-Ca²⁺ solution, and the rates of rise and fall of the response were not noticeably changed (decay time constant 1.2 ± 0.6 sec, mean rate of rise $0.0015 \Delta A_s \text{ msec}^{-1}$). Depolarizations up to $+105$ mV were not found to cause any decrease in the response size. A small shift in threshold potential for the arsenazo response to about -50 mV was apparent; in the 0-Ca²⁺ solution a shift in this direction would be expected from a surface charge effect (Costantin, 1968; Dörrscheidt-Käfer, 1976) caused by the lower concentration of divalent cations in the Ca²⁺ free solution (5 mM-Mg²⁺ as opposed to 12 mM-Ca²⁺).

External Ca²⁺ was however important for the maintenance of the Ca²⁺ transient during prolonged depolarizations. Fig. 4 shows records of arsenazo responses to long (roughly 60 sec) depolarizations to $+20$ mV from fibres bathed in Ringer's solution containing different free Ca²⁺ concentrations. The arsenazo response was maintained at close to its maximum size throughout the depolarization in 12 mM-Ca²⁺ but showed a progressively more rapid decline during the pulse when the external Ca²⁺ concen-

tration was lowered to 1.8 and 0 mM. 5 mM-MgCl₂ was included in the zero Ca²⁺ solution to stabilize the membrane, but this did not appear to alter the response, as shown by the close similarity of the records obtained in solutions containing 1.8 mM-Ca²⁺ with (Fig. 4C), and without (Fig. 4B) 5 mM-Mg²⁺.

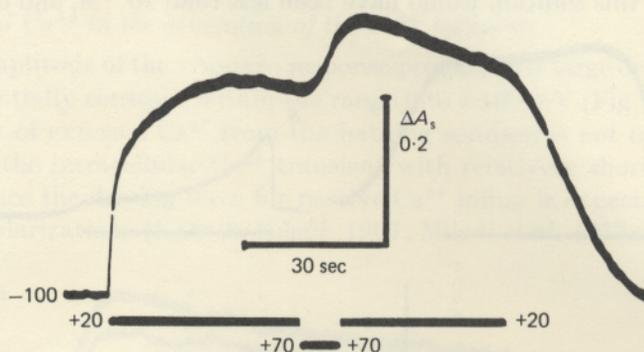


Fig. 5. Effects of an additional supramaximal depolarizing pulse during a long depolarization. The fibre was voltage clamped at a holding potential of -100 mV, and depolarized to $+20$ and $+70$ mV, as indicated by the bars. The trace is light transmission at 532–602 nm, filtered with a time constant of 33 msec. Temperature 8°C . Ringer contained 12 mM-Ca²⁺.

Possible mechanisms for the action of external Ca²⁺ on the ability of slow fibres to maintain a prolonged Ca²⁺ transient include (a) an effect on the inactivation rate of the Ca²⁺ release mechanism, and (b) a change in an influx of external Ca²⁺ ions which may be required to maintain the intracellular Ca²⁺ level. To test the importance of any Ca²⁺ influx during maintained depolarizations in 12 mM-Ca²⁺ Ringer, an additional step depolarization to $+70$ mV was given during a long depolarization to $+20$ mV (Fig. 5). This additional depolarization should have reduced any passive Ca²⁺ influx, but in all fibres examined a small rise in arsenazo signal was recorded, rather than a fall.

Latency of the arsenazo signal

In frog twitch muscle fibres a latency of a few milliseconds has been observed between the onset of a large depolarization and the initial rise of the arsenazo signal (Miledi, *et al.* 1979). We attempted to determine if a similar latency is present also in slow fibres, but because of the slow rate of rise of the arsenazo signal in these fibres we were unable to obtain clear results. Fig. 6 shows superimposed responses to a maximal 100 msec depolarizing pulse, on a fast sweep and with little filtering of the optical record. The noise on these traces is such that a latency shorter than 5–10 msec would not be detectable, but this does at least set a maximum upper limit to any latency which is present. A latency of 5–10 msec has been observed for tension development in slow fibres (Gilly & Hui, 1980), but this was at 20°C as opposed to 7.5°C in our experiment, so it seems that the onset of the arsenazo signal occurs at least as early as the development of tension.

Large voltage clamped depolarizing pulses were used in both these arsenazo and tension experiments. However, it has been reported that when slow fibres are

depolarized by a K⁺ concentration just above that required to elicit a mechanical response, there is a delay of about 15 sec before the start of tension development; the maximum tension developed is only slightly less than with higher K⁺ concentrations, and the rise in tension is rapid (less than 2 sec) following the initial latency (Lännergren, 1967*b*). We therefore performed experiments to study the time course of the arsenazo signal when fibres were voltage clamped to potentials just above the response threshold.

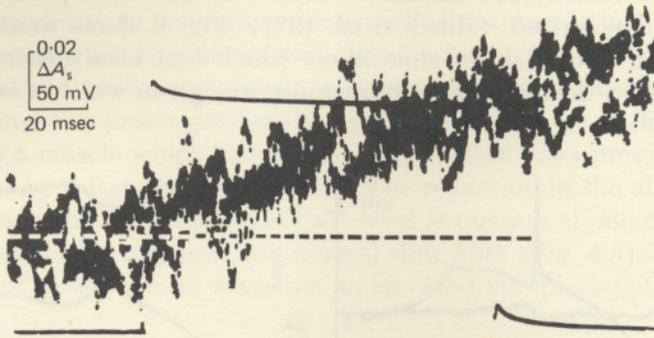


Fig. 6. Initial rise of the arsenazo signal. Superimposed records of four responses to a 100 msec duration depolarizing pulse are shown. Lower trace is membrane potential, and upper (noisy) trace is light transmission at 532–602 nm. Optical time constant was 3.3 msec, and temperature 7.5 °C. Ringer solution contained 12 mM-Ca²⁺. The dashed line indicates the baseline of the light signal.

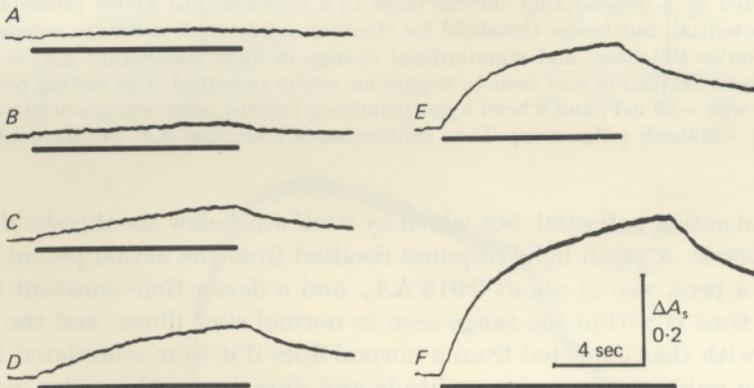


Fig. 7. Arsenazo signals in response to long depolarizations of different voltages, recorded from a denervated slow fibre (26 days after crushing the nerve; maintained at about 18 °C). The fibre was voltage clamped at a holding potential of -100 mV, and depolarized as indicated by the bars to potentials of (A) -45 mV, (B) -43 mV, (C) -41 mV, (D) -39 mV, (E) -35 mV, and (F) -27 mV. Time calibration is 4 sec, and light absorbance calibration $\Delta A_s = 0.2$. Temperature 7 °C.

These experiments were difficult to perform because of the necessity to obtain a very stable baseline level. Only in one fibre were we able to achieve good results, and this happened to be from a denervated muscle. It is probable, however, that the results would not be different from a normal fibre, and other experiments on two normal fibres suggested that they behaved similarly. Fig. 7 shows light absorbance records

in response to depolarizations to potentials between -45 and -17 mV. It is clear that the arsenazo signal was graded in size with increasing depolarizations, and that there was no apparent delay in the onset of the response at any of the potentials examined. The records also illustrate well the biphasic rate of rise of the arsenazo signal.

Denervated muscles

Following denervation, slow muscle fibres become able to respond with an action potential when depolarized. (Miledi *et al.* 1971). Fig. 8 shows arsenazo responses evoked by action potentials in slow fibres which had been denervated 28 days previously. In (A) a depolarizing current pulse was given which was just sufficient

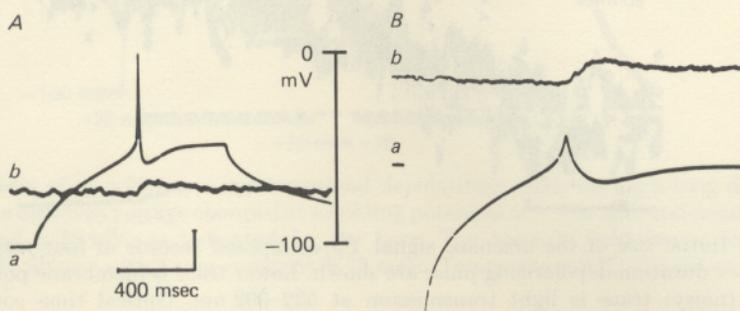


Fig. 8. Records from two slow fibres from muscles which had been denervated 26 days previously (frogs maintained at 18°C). In each frame trace a shows membrane potential, and trace b light transmission at $532\text{--}602$ nm. Temperature 7°C . A, an action potential was elicited by a depolarizing current pulse to a potential just above threshold for the action potential, but below threshold for eliciting a detectable arsenazo response. Time calibration is 400 msec, and standardized change in light absorbance $\Delta A_s = 0.045$. B, anode break excitation was used to trigger an action potential. The resting potential of the fibre was -59 mV, and a brief hyperpolarizing current pulse was given to a potential of about -200 mV (off screen). Time calibration is 1 sec, and ΔA_s calibration 0.07.

to trigger an action potential, but which by itself was below the threshold for eliciting a light response. A small light response resulted from the action potential however, which had a peak size of about $0.015 \Delta A_s$, and a decay time constant of $0.5\text{--}1$ sec. The decay time is within the range seen in normal slow fibres, and the peak size is consistent with that expected from a normal fibre if it were stimulated by a passive depolarizing pulse of the same amplitude and duration as the action potential.

Fig. 8B shows a response from another slow fibre in the same muscle, in which an action potential was elicited by anode break excitation. The fibre was held at a potential of -55 mV by adjusting the current through the dye pipette, and then briefly hyperpolarized to about -200 mV. An action potential was triggered during recovery from hyperpolarization, and gave rise to a small light response. Again, the time course and size of this response corresponds to those seen in normal fibres depolarized by pulses with the same time course and magnitude as the action potential.

Light responses to brief (100 msec) depolarizing pulses under voltage clamp were found to be essentially unchanged by denervation of the muscle (Fig. 9A), and the relationship between depolarizing potential and peak size of the light response was

as in normal fibres (Fig. 9B). Denervated slow fibres also showed a maintained response during prolonged depolarizations in 12 mM-Ca²⁺ Ringer, which was not noticeably different from normal fibres (Fig. 9C).

Reinnervated muscles

During the early phase of reinnervation, slow muscle fibres become transiently reinnervated by fast conducting axons (Elul *et al.* 1970; Schmidt & Stefani, 1976, 1977), and their ability to maintain a prolonged contracture is lost (Elul *et al.* 1970; Lehman & Schmidt, 1979; Schmidt, 1980). We have found that there is a corresponding failure in the maintained arsenazo response during long depolarizations. Fig. 10 shows the light response to a prolonged depolarization under voltage clamp, recorded from a slow fibre in a muscle which had been denervated 36 days previously. This fibre was innervated by a fast conducting axon. The reduction in the ability of the fibre to maintain an elevated myoplasmic Ca²⁺ level is obvious if one compares Fig. 10 with the corresponding response in a normal slow fibre (Fig. 4A).

The ability of slow fibres to maintain an elevated myoplasmic Ca²⁺ level during

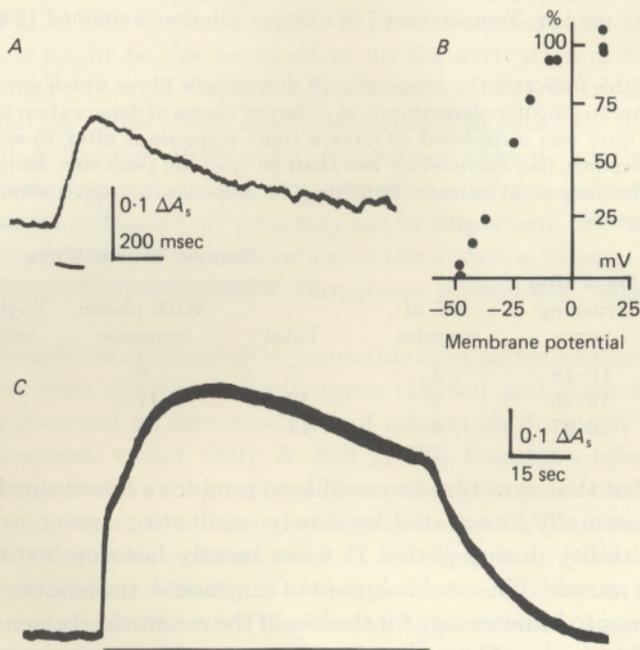


Fig. 9 Arsenazo responses from denervated slow fibres under voltage clamp. Muscles had been denervated from 10 to 26 days previously, and all the fibres illustrated had action potentials. Ringer solution contained 12 mM-Ca²⁺. Temperature 7 °C, and optical time constant 33 msec for all experiments. *A*, light transmission signal at 532–602 nm in response to a 100 msec depolarizing pulse to 0 mV (indicated by bar). *B*, voltage dependence of the arsenazo signal elicited by depolarizing pulses of various sizes. Abscissa, membrane potential during the pulse; ordinate, peak size of the light response. Data are from one fibre, and were obtained and calculated as in Fig. 2. *C*, arsenazo response to a long (approx. 80 sec) depolarization to +20 mV. The fibre was voltage clamped at a holding potential of -100 mV, and depolarized for the time indicated by the bar. The inflexion in the rising phase may be a movement artifact. Same fibre as Fig. 8A.

prolonged depolarization at various periods of reinnervation is summarized in Table 1. In period I all fibres were denervated, and muscles showed no response to nerve stimulation. Period II represents the early reinnervation phase, when slow fibres would be mostly innervated by fast conducting axons, and period III is the late reinnervation phase, when slow fibres were largely innervated by slowly conducting axons.

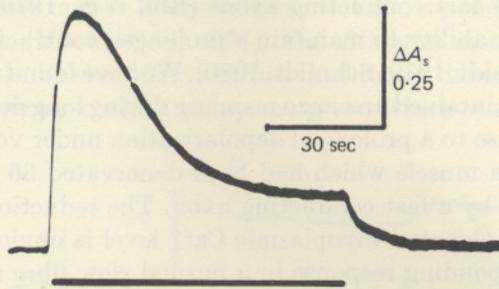


Fig. 10. Arsenazo response to a long depolarization, recorded from a slow fibre during the early reinnervation phase. The fibre was depolarized from -100 to $+20$ mV for the time indicated by the bar. Temperature 7°C . Ringer solution contained 12 mM-Ca^{2+} .

TABLE 1. The Table indicates the proportion of slow muscle fibres which gave a maintained (tonic) arsenazo response to long depolarizations, at different stages of denervation and reinnervation. For this analysis a fibre was considered to have a tonic response if after 75 sec of depolarization to $+20$ mV, the response had declined by less than 50% of the peak size. Ringer solution contained 12 mM-Ca^{2+} . The degree of muscle twitching in response to nerve stimulation was assessed qualitatively by eye

Period	Days after crushing nerve	No. of muscles	Number of slow fibres			Twitching to nerve stimulation
			Total	With phasic response	With tonic response	
I	15-18	4	5	0	5	none
II	19-25	2	12	11	1	slight
III	26-37	6	17	3	14	marked

It is apparent that slow fibres were able to produce a maintained arsenazo response when either normally innervated by slowly conducting axons, or when denervated, but lost this ability during period II when mostly fast conducting axons would be present in the muscle. The establishment of functional, transmitting synapses did not, however, appear to be necessary for the loss of the maintained arsenazo response. From a total of fourteen slow fibres showing only a transient response, five were found in which nerve stimulation did not elicit an end-plate potential. A similar loss of maintained contracture before neuromuscular transmission is restored has been previously observed (Elul *et al.* 1970).

DISCUSSION

Excitation/contraction coupling in slow fibres

Our results show that the myoplasmic Ca^{2+} transient in activated slow muscle fibres, as detected with arsenazo III, has many properties in common with that in twitch fibres (Miledi *et al.* 1977a, 1979). Specifically, (i) a sigmoid relationship was

found between membrane depolarization and the size of the arsenazo response, (ii) reduction of the external Ca²⁺ concentration to very low levels with EGTA did not reduce the arsenazo response to short depolarizing pulses, (iii) the latency between the onset of a large depolarizing pulse and the beginning of the arsenazo response was short (less than 5–10 msec), (iv) depolarizing pulses to high positive potentials, which would be expected to reduce any influx of external Ca²⁺, did not reduce the size of the response to short pulses, even in fibres bathed in the low-Ca²⁺ solution. These findings indicated that an influx of Ca²⁺ ions from the bathing medium during short depolarizations does not directly contribute to the intracellular Ca²⁺ transient, and support the view that contractile activation in slow fibres involves Ca²⁺ release from internal stores in a manner similar to that found in twitch fibres (Franzini-Armstrong, 1973; Flintney, 1971; Costantin *et al.* 1967; Miledi *et al.* 1977a; Gilly & Hui, 1980).

External Ca²⁺ is, however, important in determining the ability of slow fibres to maintain a Ca²⁺ transient during prolonged depolarization. At the end of a 1 min depolarization to +20 mV the arsenazo signal had declined to about 90% of the peak value in fibres bathed in 12 mM-Ca²⁺ Ringer, 20% in 1.8 mM-Ca²⁺ Ringer, and 5% in Ca²⁺ free Ringer (Fig 3.) This decline in ability to produce a maintained response when external Ca²⁺ is low might be due to an effect on the activation of the excitation/contraction coupling process (cf. Caputo & De Bolaños, 1979), or to the reduction of an influx of external Ca²⁺ ions which might be required to maintain the internal free Ca²⁺ level (Stefani & Uchitel, 1976). The observation that depolarization to high positive potentials during maintained depolarizations did not reduce the arsenazo signal suggests that the influx of Ca²⁺ ions may not be important. We have previously observed effects of external Ca²⁺ concentration on the duration of long Ca²⁺ transients in twitch muscle fibres which were similar, though less pronounced, than in slow fibres (Miledi *et al.* 1979).

Previous reports of effects of external Ca²⁺ concentration on maintained contractures in slow fibres have been equivocal; Lännergren (1976a) and Nasledov, Zachar & Zacharova (1966) observed an increased rate of relaxation during K⁺ contractures when Ca²⁺ was lowered, whilst Gilly & Hui (1980) found no effect of 0–Ca²⁺ solution on contractures elicited by voltage clamped depolarizations. The prolonged 'tail' of the arsenazo response which we observed in low Ca²⁺ solutions could help explain this discrepancy; this residual level of free Ca²⁺ may have been sufficient to activate the contractile machinery in the experiments of Gilly & Hui.

The maximum rates of rise and decline of the arsenazo signal elicited by supramaximal voltage clamped depolarizing pulses were both roughly 20 times slower in slow than in twitch muscle fibres (compare for example Fig. 1 of this paper and Fig. 3 of Miledi *et al.* 1977a), although the maximum size of the response during long pulses was similar in the two fibre types (Miledi *et al.* 1979). Data on the rates of tension development and relaxation in slow fibres at 7 °C are not available, so we cannot directly compare the time courses of contraction with our data on the intracellular Ca²⁺ transient. An approximate comparison is, however, provided by the observation that both contraction and relaxation of slow fibres at room temperature occur 6 times more slowly than for twitch fibres (Lännergren, 1978; Lehman & Schmidt, 1979). The time course of the intracellular Ca²⁺ transient in slow fibres might therefore be sufficiently slow to account by itself for the slow time courses

of tension development and relaxation, although the rate of tension development by the myofibrils (Costantin *et al.* 1976); and possibly other stages in the excitation/coupling process (Gilly & Hui, 1980) could also be important.

Prolonged contractions of slow muscle fibres might involve some recycling of internal Ca^{2+} , via sarcoplasmic reticulum release and re-uptake. A number of observed features of the Ca^{2+} transients would tend to reduce the metabolic expenditure on this process. (i) The rates of release and re-uptake are slow, (ii) with long responses the rate of decline of the arsenazo signal becomes linear suggesting that re-uptake reaches a maximum limiting rate, and (iii) the rise of the transient shows an initial rapid increase, followed by a slower rate of rise. This last factor might allow a contraction to be developed fairly rapidly, but then maintained by only a low rate of Ca^{2+} release.

Ca²⁺ transients in denervated and reinnervated slow fibres

We did not observe any significant changes from the normal in any of the parameters of the Ca^{2+} transient studied in denervated slow fibres. Action potentials were present in these fibres and gave rise to small arsenazo signals, but the size of these was not different from that expected if the same depolarization were applied to a normal slow fibre. These findings are in agreement with the observations of Elul *et al.* (1970) and Lehmann & Schmidt (1979), that contractile responses of slow fibres were not affected by denervation.

During the early phase of reinnervation the ability of slow fibres to give a maintained arsenazo response in 12 mM- Ca^{2+} Ringer was reduced. A similar effect has previously been noted on maintained contractures (Elul *et al.* 1970; Lehman & Schmidt, 1979). During this phase of reinnervation the fast conducting axons (but not the slow) have reached the muscle, and begin to form synaptic contacts with both the twitch and slow fibres. As with the contractures (Elul *et al.* 1970), the establishment of functionally transmitting synapses in a slow fibre was not a necessary condition for the decrease in maintained response to occur. Therefore, it seems that the fast axons may influence the depolarization induced Ca^{2+} release from the sarcoplasmic reticulum via a 'trophic' effect which is independent of synaptic activity similar to the action of motor axons on acetylcholine sensitivity (Miledi, 1960) and of slowly conducting axons on the action potential of slow fibres (Schmidt & Stefani, 1977). The mechanism of this effect is not clear, but since the characteristic electron-microscopic structure of slow muscle fibres is not altered when the muscle fibres are innervated by fast axons (Miledi & Orkand, 1966) it seems likely that the change in Ca^{2+} transient involves a change in the rate of inactivation of the coupling process between depolarization of the muscle membrane, and Ca^{2+} release from the sarcoplasmic reticulum (Caputo & De Bolños, 1979).

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