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CALCIUM TRANSIENTS EVOKED BY ACTION POTENTIALS IN FROG TWITCH MUSCLE FIBRES

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SUMMARY

1. Intracellular Ca^{2+} transients were recorded from frog twitch muscle fibres in response to action potentials and repetitive stimulation, using ionophoretically injected arsenazo III as a Ca^{2+} monitor. A dual wave-length optical system was used to measure absorbance changes of the injected dye from small areas of single fibres within the cutaneous pectoris muscle.

2. The absorbance spectrum of the injected arsenazo III in a resting fibre was consistent with an intracellular free Mg^{2+} level of a few hundred micromolar, assuming an intracellular pH of 7·1. The resting free Ca^{2+} concentration was below the limit of resolution.

3. The wave-length dependence of the arsenazo light absorbance signal during twitches followed that expected for Ca^{2+} binding to the dye. Recordings made at wave-lengths where the dye is maximally sensitive to pH or Mg^{2+} concentration changes indicated that interference from these sources is minimal at the usual wave-length pair (650–700 nm) used for Ca^{2+} recordings.

4. Over a wide range of intracellular dye concentrations, the size of the arsenazo response to an action potential increased linearly with dye concentration (100–1000 μM), although there were deviations from this relationship at low and high concentrations.

5. An approximate estimate of 8 μM was obtained for the peak free Ca^{2+} concentration change following a single action potential. Changes in temperature (6–25 °C) did not significantly affect the size of the free Ca^{2+} transient. During maximal tetanic stimulation the signal rose to about three times higher than the twitch response. An approximate minimum estimate of the increase in total cytoplasmic Ca^{2+} concentration during a twitch gave a value of 220 μM.

6. A latency of about 1·5 ms (at 10 °C) was observed between the foot of an action potential and the onset of the arsenazo response. Recordings made using a narrow measuring light slit, placed either at the edge or the centre of a fibre, suggested that only a small part of this latency could be due to inward conduction of the action potential along the T-tubules.

7. The decay phase of the arsenazo response to an action potential followed an exponential time course, with a time constant of 71 ms at 10 °C. This time constant was strongly temperature-dependent, with a Q_{10} of about 2·4. An Arrhenius plot of the decay time constant gave a straight line.

8. During repetitive stimulation, the arsenazo responses evoked by successive impulses showed two changes: a progressive decrease in amplitude and a slowing of the decay. The extent to which successive responses summated during a tetanus depended upon the balance between these two effects.

INTRODUCTION

It is now generally accepted that intracellular Ca^{2+} ions play a key role in excitation–contraction coupling in skeletal muscle (for recent review see; Ebashi, 1976; Endo, 1977; Costantin, 1975; Caputo, 1978; Stephenson, 1981). In brief, changes in potential across the T-system membrane are coupled by a poorly understood mechanism (Schneider, 1981) to cause release of stored Ca^{2+} ions from the sarcoplasmic reticulum (s.r.) into the muscle cytoplasm. The resulting rise in free cytoplasmic Ca^{2+} concentration, from a low resting level, initiates contraction by means of Ca^{2+} binding to troponin in the thin filaments. Relaxation is finally brought about by active re-uptake of Ca^{2+} into the s.r.

Studies of excitation–contraction coupling have been greatly facilitated by the development of techniques for monitoring changes in intracellular free Ca^{2+} during contraction. The first of these measurements were made using the indicator dye Murexide (Jöbsis & O'Connor, 1966), but this is very insensitive and all subsequent work has used either the Ca^{2+}-dependent photoprotein aequorin (Blinks, Rudel & Taylor, 1978; Eusebi, Miledi & Takahashi, 1980), or the metallochromic indicator dyes arsenazo III and antipyrylazo III (Miledi, Parker & Schalow, 1977*a*, *b*, *c*, 1981; Miledi, Nakajima, Parker & Takahashi, 1981; Kovács, Rios & Schneider, 1979; Baylor, Chandler & Marshall, 1979*a*). Both of these latter two techniques offer their own specific advantages and disadvantages, which have been considered by several authors (Blinks, Prendergast & Allen, 1976; Ashley & Campbell, 1979; Grinnell & Brazier, 1981; Stinnakre, 1980; Thomas, 1982). Because each of the techniques present generally different disadvantages and problems of interpretation, it seems sensible to corroborate findings with both methods where possible.

We have used arsenazo III as an intracellular Ca^{2+} indicator to study Ca^{2+} transients following action potentials in frog twitch muscle fibres. In this paper we describe some of the characteristics of the dye when used for this purpose, and explore some of the properties of the Ca^{2+} transients. Preliminary reports of some results have been presented (Miledi, Parker & Schalow, 1977*a*, *c*).

METHODS

Preparation

Experiments were performed on the cutaneous pectoris muscle of *Rana temporaria*. The muscle was mounted in a glass bottomed chamber and held by stainless steel hooks. To reduce movement artifacts during contraction the muscle was usually stretched to a striation spacing of about 3·7 μm. Peltier elements in the base of the chamber allowed the temperature to be controlled and this was measured by a thermistor placed close to the muscle. Unless otherwise stated, experiments were performed at 9–11 °C. The normal bathing solution contained (in mM): NaCl, 120; KCl, 2; $CaCl_2$, 1·8; HEPES, 5, at pH 7·2. 0 Ca^{2+} solution contained: NaCl, 120; KCl, 2; $MgCl_2$, 5; EGTA, 1; HEPES, 5, at pH 7·2.

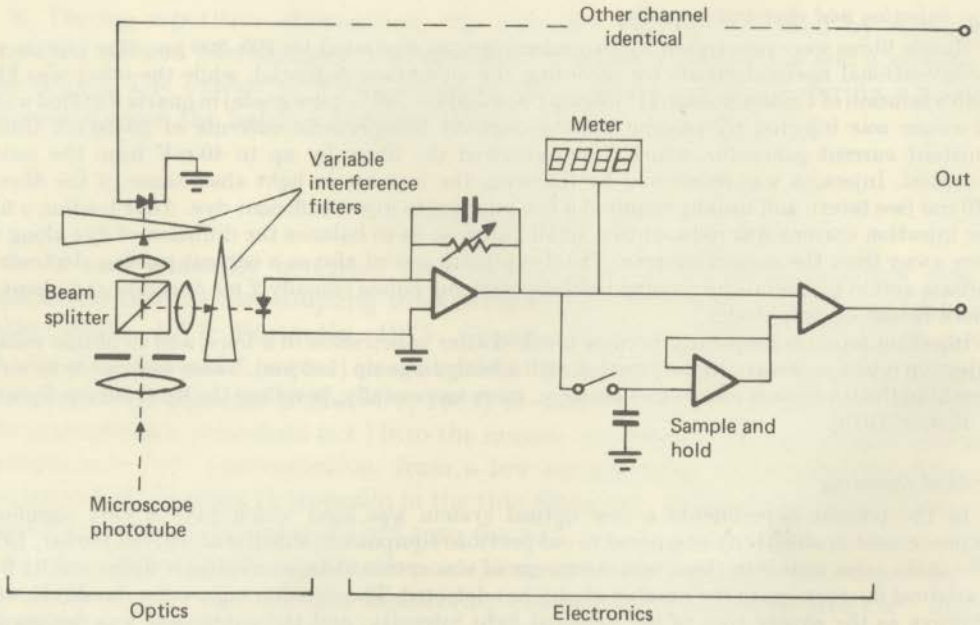


Fig. 1. Optical system and electronic circuit for recording light absorption changes in muscle fibres. See text for details.

In this paper arsenazo III signals are expressed in terms of either ΔA or $\Delta A/A_{570}$. The first, ΔA, is absorbance change, namely $\Delta A \approx \Delta I/(2{\cdot}3\, I)$, in which ΔI is the change in light intensity emerging from the fibre caused by the stimulation, and I is the intensity emerging from the resting fibre. This approximation holds because in the present recordings $\Delta I \ll I$, and for this case $\ln(\Delta I/I) \approx \Delta I/I$. The factor of 2·3 ($\approx \ln 10$) is used to convert to absorbance units to base 10. The term A_{570} is the resting absorbance of the injected dye at 570 nm; namely $A_{570} = -\log_{10}(I_{570}/I_{0,570})$, in which I_{570} is the intensity of light emerging from the fibre after dye injection and $I_{0,570}$ is the light emerging from the fibre before dye injection.

RESULTS

Injection of arsenazo III into muscle fibres

Injection of arsenazo into a fibre was monitored by following the decrease in light transmission through the fibre at 570 nm, a wave-length where the dye has a high absorbance, but little sensitivity to Ca^{2+}, Mg^{2+} or pH. Fibres were generally injected to give a decrease in light transmission of about 0·2. Several minutes were usually required for injection, and after this time the concentration of dye in the fibre appeared by eye to be uniform over the area of the measuring slit.

The absorption spectrum of the injected dye was measured from two fibres at rest (Fig. 2). For comparison, absorption spectra were obtained also for calibrating solutions of arsenazo contained in a glass capillary which replaced the muscle fibre. The spectrum of the dye in the muscle deviates slightly from that expected from arsenazo III in a solution containing 120 mM-KCl at a pH of 7·1, but a closer fit would be obtained by addition of about 0·5 mM-Mg^{2+}. There are no significant peaks at 600 and 650 nm in the muscle spectra, indicating that any Ca^{2+}–dye complex present is below the limit of resolution.

Dye injection and electrical recording

Muscle fibres were penetrated by two micropipettes separated by 100–300 μm. One pipette was a conventional micro-electrode for recording the membrane potential, while the other was filled with a solution of 1 mM-arsenazo III (Sigma Chemical Co.; 98 % pure grade) in quartz distilled water. Arsenazo was injected by passing pipette negative ionophoretic currents of 20–50 nA from a constant current generator, which hyperpolarized the fibres by up to 40 mV from the resting potential. Injection was monitored by following the increase in light absorbance of the fibre at 570 nm (see later), and usually required a few minutes to inject sufficient dye. After loading a fibre the injection current was reduced to a small value, so as to balance the diffusion of dye along the fibre away from the measuring area. The dye pipette served also as a current passing electrode to initiate action potentials by passing brief depolarizing pulses (usually 2 ms duration and about 1·5 times threshold amplitude).

Injection pipettes frequently became blocked after penetration of a fibre, and to obtain reliable injection it was necessary to use pipettes with a fairly large tip (1–2 μm). These were made by either breaking the tip against connective tissue or, more successfully, bevelling the tip (Lederer, Spindler & Eisner, 1979).

Optical recording

In the present experiments a new optical system was used which gave a very significant improvement in sensitivity compared to our previous equipment (Miledi *et al.* 1977*a*; Parker, 1979). The main noise source in these measurements of absorption changes in muscle fibres results from statistical fluctuations in the number of photons detected. The signal to noise ratio should therefore improve as the square root of the detected light intensity, and the equipment was designed to maximize both the incident light flux through the muscle fibre and the quantum efficiency of the light detectors.

The optical system was based around a Zeiss Ergaval microscope. Light from a 100 W quartz halogen lamp, operated from a well stabilized power supply, was focused onto the muscle by a long working distance condenser. To restrict the measuring light to the desired portion of a single fibre, a Zeiss variable slit diaphragm was placed at the field stop of the microscope and an image of this focused onto the fibre by the condenser. Unless otherwise stated the rectangular slit was adjusted so as just to cover the width of the fibre and with a length equivalent to about three fibre diameters. It was positioned to include the tip of the dye injection pipette. A dichroic heat filter blocking wave-lengths above 750 nm was placed below the condenser, both to minimize heating of the muscle and to act as a band stop filter for the variable interference filters in the detector head.

Light transmitted through the muscle was collected by a Leitz 25X long working distance objective (N.A. = 0·22). This lens was chosen because of its great convenience when working with micro-electrodes. Water immersion objectives with higher numerical aperture were found to give slightly better optical recordings, but were very inconvenient. Artifacts in light transmission due to ripples on the surface of the bathing fluid were minimized by using a low fluid level above the muscle, and by mounting the equipment with good vibration isolation. Light from the objective lens could be switched for either viewing through the eyepieces, or for measurement by the detector head mounted on the microscope phototube.

Fig. 1 shows a schematic diagram of the optics and electronics of the detector system. A beam splitting cube divided the light into two similar paths and lenses focused spots of about 1 mm diameter onto two low noise photodiodes (E.G. & G. Inc. – PV 100A). In front of each photodiode a continuously variable interference filter (Oriel Scientific – filter monochromator) allowed selection of any desired wave-length between 400 and 700 nm. Under the conditions used, the half peak band width of these filters would be about 13 nm at 550 nm and 16 nm at 650 nm (manufacturers specifications). The wave-length scales of the filters were calibrated against known fixed interference filters.

Virtual earth circuits were used to measure the photocurrents from the diodes, and gave a linear measure of the incident light. Signals were recorded differentially from the two channels on an oscilloscope, after automatic subraction of base line offsets by means of sample and hold circuits and filtering by simple RC circuits. Digital meters allowed the total light intensity from each channel to be monitored and the gain of one channel was variable, so that differences in photodiode sensitivity and light absorption by the muscle at different wave-lengths could be compensated.

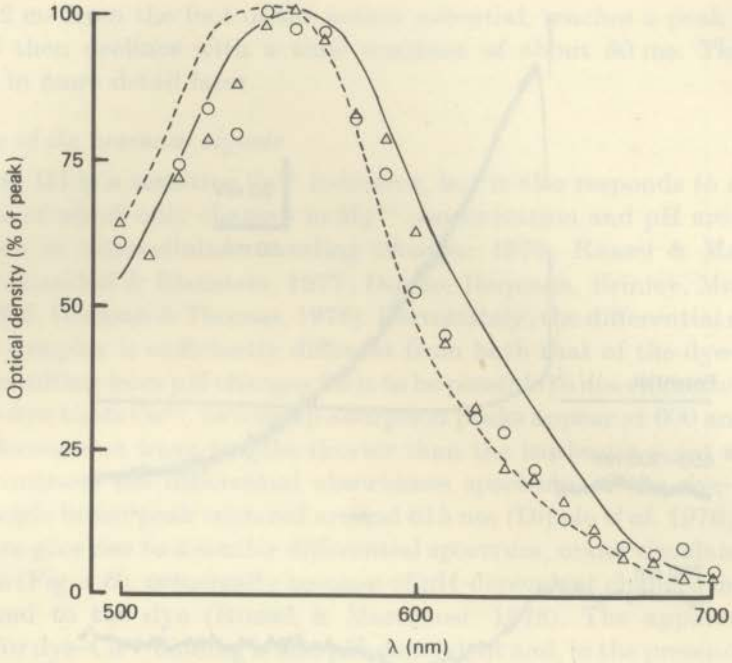
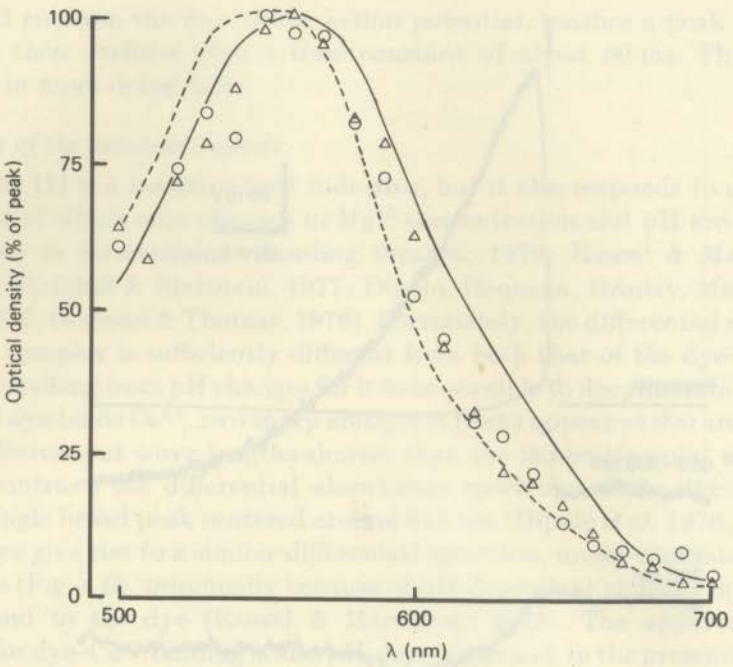


Fig. 2. Absorbance spectrum of arsenazo III injected in resting muscle fibres. Data from two fibres (△ and ○) are shown. Measurements were taken from the fibre of light transmission at 10 nm increments, firstly before injecting dye and then after injection. Light transmission at 570 nm was continuously monitored on a second channel to confirm that the incident light intensity and the intracellular dye concentration did not change significantly during the measuring period. The optical density of the injected dye at each wave-length (A_λ) was calculated from $A_\lambda = -\log_{10}(I_\lambda/I_{0,\lambda})$; (see Methods). Measurements have been normalized by expressing as a percentage of the peak absorbance. The two curves are tracings of absorbance spectra measured in the same way for calibrating solutions of arsenazo III contained in a glass capillary (internal diameter 110 μm). Composition of the solution was (in mM): KCl, 120; arsenazo III, 1; EGTA, 0·1; HEPES, 5; at pH 7·1. The dashed curve was obtained with this solution, whilst the continuous curve was obtained after addition of 1 mM-$MgCl_2$.

The level of free Mg^{2+} in the calibrating solution required to approximate the muscle spectrum will depend upon the pH, since the apparent affinity constant of arsenazo for Mg^{2+} is lower at more acid pH. We used a pH value of 7·1, which appears to be representative of the intracellular pH in frog muscle (Roos & Boron, 1981). Baylor *et al.* (1979*a*) have previously reported that the resting spectrum of arsenazo III in muscle is consistent with 1 mM-free Mg^{2+}, but this was at a pH of 6·9, so there would appear to be little discrepancy between these results.

Arsenazo responses elicited by action potentials

Records of intracellular Ca^{2+} transients were obtained from arsenazo III-loaded fibres by measuring differentially at the wave-length pair 650–700 nm. Arsenazo shows a large Ca^{2+}-dependent increase in absorption at 650 nm, but little change at 700 nm. Subtraction of simultaneous signals at these wave-lengths therefore allows recording of Ca^{2+} transients with a substantial cancellation of any movement artifacts, which tend to affect both wave-lengths approximately equally.



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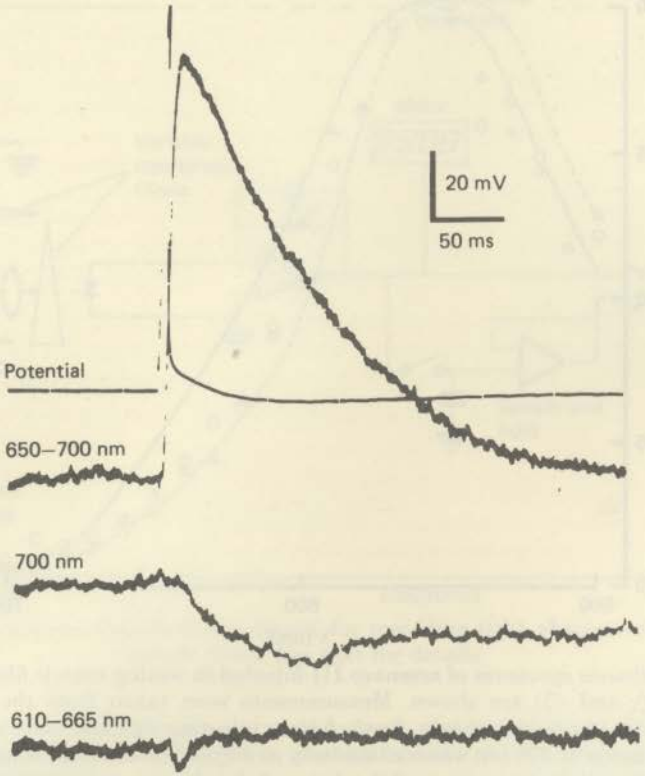

Fig. 3. Light absorbance changes from an arsenazo III-loaded muscle fibre in response to an action potential. Traces show respectively from top to bottom: membrane potential, light transmission at the wave-length pair 650–700 nm, transmission at 700 nm, and transmission at 610–665 nm. The 650–700 nm trace monitors Ca^{2+}-dependent changes in arsenazo absorption, the 700 nm trace gives a monitor of the movement artifact, and the 610–665 nm trace indicates pH- or Mg^{2+}-concentration-dependent changes in dye absorption. The fibre was stimulated by a just supra-threshold depolarizing pulse of 3 ms duration. Resting potential was −75 mV. Optical records were low pass filtered by a simple RC circuit with a cut-off frequency of 500 Hz. The calibration bar corresponds to a light absorption change of $\Delta A = 0{\cdot}0015$ for all optical records. Dye was injected to give a resting absorbance at 570 nm of $A_{570} = 0{\cdot}096$. Temperature 9 °C. In this and all following Figures, an increase in light absorption (by the first wave-length listed for a differential pair) corresponds to an upward deflection.

The changes in light absorption which accompany an action potential in a loaded fibre are illustrated in Fig. 3. A large transient was seen at the wave-length pair 650–700 nm, whilst at 700 nm alone a smaller signal with a different time course was observed. The 700 nm response probably originated from movement during contraction. It began after the rising phase of the 650–700 nm trace and was reduced in muscles which were stretched more strongly. This component will be almost completely cancelled out in the differential 650–700 nm trace, and differential records from fibres which had not been injected with arsenazo usually showed no detectable signals to an action potential (e.g. first optical record in Fig. 5*A*).

At a temperature of 10 °C the arsenazo signal at 650–700 nm begins with a latency

of about 2 ms from the foot of the action potential, reaches a peak after a further 8 ms and then declines with a time constant of about 80 ms. These phases are discussed in more detail later.

Specificity of the arsenazo signals

Arsenazo III is a sensitive Ca^{2+} indicator, but it also responds to a wide range of other ions, of which only changes in Mg^{2+} concentration and pH are likely to cause interference in intracellular recording (Scarpa, 1979; Russel & Martonosi, 1978; Kendrick, Ratzlaff & Blaustein, 1977; Dipolo, Requena, Brinley, Mullins, Scarpa & Tiffert, 1976; Gorman & Thomas, 1978). Fortunately, the differential spectrum of the dye–Ca^{2+} complex is sufficiently different from both that of the dye–Mg^{2+} complex and that resulting from pH changes for it to be possible to discriminate between them. When the dye binds Ca^{2+}, two sharp aborption peaks appear at 600 and 650 nm, with a broad decrease at wave-lengths shorter than the isosbestic point at 570 nm (Fig. 4*B*). In contrast, the differential absorbance spectrum of the dye–Mg^{2+} complex shows a single broad peak centered around 615 nm (Dipolo *et al.* 1976; Scarpa, 1979). pH changes give rise to a similar differential spectrum, under simulated intracellular conditions (Fig. 4*B*), principally because of pH-dependent changes in the amount of Mg^{2+} bound to the dye (Russel & Martonosi, 1978). The apparent dissociation constant for dye–Ca^{2+} binding is also pH-dependent and, in the presence of Ca^{2+}, small pH changes will give absorbance changes indistinguishable from those produced directly by changes in Ca^{2+} concentration (Ahmed, Kragie & Connor, 1980). This effect is unlikely to be directly significant in muscle, since the resting free Ca^{2+} level is very low (Blinks *et al.* 1978). However, a pH transient occurring during a Ca^{2+} transient could distort the time course of the absorbance signal.

To examine any possible pH- or Mg^{2+}-dependent contribution to the arsenazo signals following an action potential, we examined the wave-length-dependence of the signal in two ways. The first was to record from fibres differentially at the wave-length pair 610–665 nm. Ca^{2+}-dependent changes of dye absortion are closely similar at these two wave-lengths, and should cancel out on differential recording, whilst pH or Mg^{2+} changes will give rise to a large absorbance change (see arrows in Fig. 4*B*). The lowest trace in Fig. 3 shows a typical response at 610–665 nm. Similar records were obtained from six other fibres examined. A small, brief, downward deflection is seen (corresponding to a decrease in absorption at 610 nm relative to 665 nm) which begins with a similar latency to the 650–700 nm signal, but returns to the base line at about the time the 650–700 nm trace reaches a peak. The remainder of the trace is essentially flat. At present the origin of this brief signal is not clear; one possibility is a rapidly buffered acidification of the fibre during the release of Ca^{2+} by the s.r. Whatever the origin of the signal, it appears that the Ca^{2+}-dependent record at 650–700 nm is contaminated only very slightly (< about 5%) by pH or Mg^{2+} changes

A second test was to measure the wave length-dependence (spectrum) of the arsenazo signal to an action potential. Previous measurements have been made using a limited number of fixed interference filters (Baylor *et al.* 1979*a*; Miledi *et al.* 1981*a*), but the use of variable filters in the present equipment allows a much better resolution. The mean spectrum measured from two fibres is shown in Fig. 4*A*. This

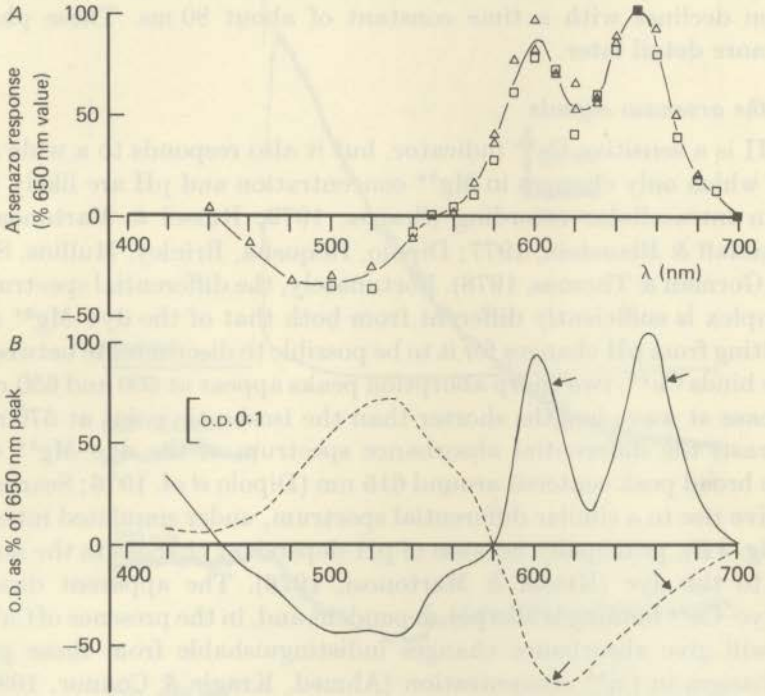

Fig. 4. Wave-length-dependence of arsenazo III absorbance changes. *A*, arsenazo signals recorded from loaded muscle fibres in response to action potentials. Results from two fibres are shown (△ and □). Signals were obtained by altering the wave-length of one recording channel in 10 nm increments and recording differentially with respect to the second channel set at 700 nm. A separate response was elicited for measurements at each wave-length. The recording gain on the variable channel was adjusted to compensate for differing sensitivities of the photodiode at different wave-lengths. The peak amplitudes of arsenazo signals changed gradually during the experiments, probably because of changes in intracellular dye concentration. This effect was corrected as follows: measurements at several wave-lengths were bracketed by measurements at 650 nm and the values were expressed as a percentage of these control measurements. The values at 650 and 700 nm are hence by definition respectively 100 % and 0 % (■). The curve is drawn by eye through the mean values at each wave-length. *B*, tracings of spectrophotometer measurements showing arsenazo III difference spectra for changes in Ca^{2+} concentration (continuous curve) and changes in pH (dashed curve). Measurements were made on a Pye Unicam SP 1800 spectrophotometer, with a band width of 1 nm. The basic composition of the solution was (in mM): arsenazo III, 0·1; KCl, 120; $MgCl_2$, 2; HEPES, 5; EGTA, 1. pH was adjusted to 7 (except for the pH 6·75 solution) before each measurement. This solution was placed in both cuvettes of the double beam spectrophotometer, and gave an optical density of 2·6 at 570 nm with a 1 cm path length. The continuous curve shows the difference spectrum obtained after adding 1 mM-$CaCl_2$ to one cuvette. This concentration of Ca^{2+} gave an absorption change which was about half maximal. The dashed curve shows the difference spectrum after altering the pH of one cuvette to 6·75, with both cuvettes containing no added Ca^{2+}. Arrows indicate the wave-length pair (610–665 nm) selected in the lowest trace of Fig. 3 to give maximal sensitivity to pH changes, and minimal sensitivity to Ca^{2+} concentration changes.

is qualitatively similar to the *in vitro* difference spectrum for arsenazo-Ca^{2+} measured on a spectrophotometer (Fig. 4*B*). The differences which do exist (principally the shallower trough at 630 nm and the shift in isosbestic point) are largely attributable to the wider bandwidth in the muscle recordings.

Baylor, Chandler & Marshall (1979*b*) have reported that, in addition to signals due to Ca^{2+} binding, arsenazo III shows also a dichroic component in recordings from muscle fibres, which is more prominent if the measuring light is polarized at 90° to the fibre long axis than at 0°. Under our recording conditions we have not observed any significant differences in the signal recorded at 650–700 nm with different orientations of polarized light. This is probably because the dichroic component is expected to be small in comparison to the Ca^{2+} component at these wave-lengths and with the relatively high dye concentrations which we used.

We believe therefore that the action potential induced arsenazo signal at 650–700 nm reflects almost entirely a change in intracellular Ca^{2+} concentration.

Influence of amount of dye injected

Arsenazo III binds Ca^{2+} with a high affinity and is injected to quite large intracellular concentrations (around 1 mM) for recording. It is possible, therefore, that the presence of dye in the muscle may significantly alter the changes in free Ca^{2+} concentration occurring in the muscle fibre. To examine the extent of this disturbance we recorded arsenazo responses to action potentials during gradual injection of increasing amounts of dye. These experiments served also to determine the relationship between dye concentration and the response elicited by a (presumed) fixed change in free Ca^{2+} level in the fibre.

At present, the stoichiometry of arsenazo III–Ca^{2+} binding is controversial, and may depend upon the particular conditions of use. Some reports (Dipolo *et al.* 1976; Scarpa, Brinley, Tiffert & Dubyak, 1978; Ahmed *et al.* 1980) suggest a 1 : 1 stoichiometry (which would give a linear response relationship with dye concentration), whilst others (Thomas, 1979) suggest a 2 : 1 relationship (giving a square law relationship), or even a mixture of 2 : 1 and 2 : 2 complexes (Palade & Vergara, 1981). Recently, Brown & Rydqvist (1981) have suggested that those findings may be explained by 1 : 1 binding, but with an apparent dissociation constant which decreases with increasing arsenazo III concentration.

Fig. 5*A* shows sample records obtained whilst injecting a fibre with arsenazo III. The traces were obtained at the wave-length pair 650–570 nm, so that the change in absorbance of the fibre at 570 nm could be used as a measure of the intracellular dye concentration. At both 570 and 700 nm, arsenazo shows only very small Ca^{2+}-dependent changes in absorption, but the 700 nm wave-length was generally found to be better for cancellation of movement artifacts. During injection of increasing amounts of dye the size of the response first increases and then begins to fall. The time course of decay of the response also becomes slower with large amounts of dye.

These changes are shown in Fig. 5*B*, *C*, plotted against the estimated intracellular concentration of arsenazo. Similar results were obtained from six other fibres examined. Over a large part of the concentration range covered, the size of the response increases linearly with dye concentration. However, at levels above about

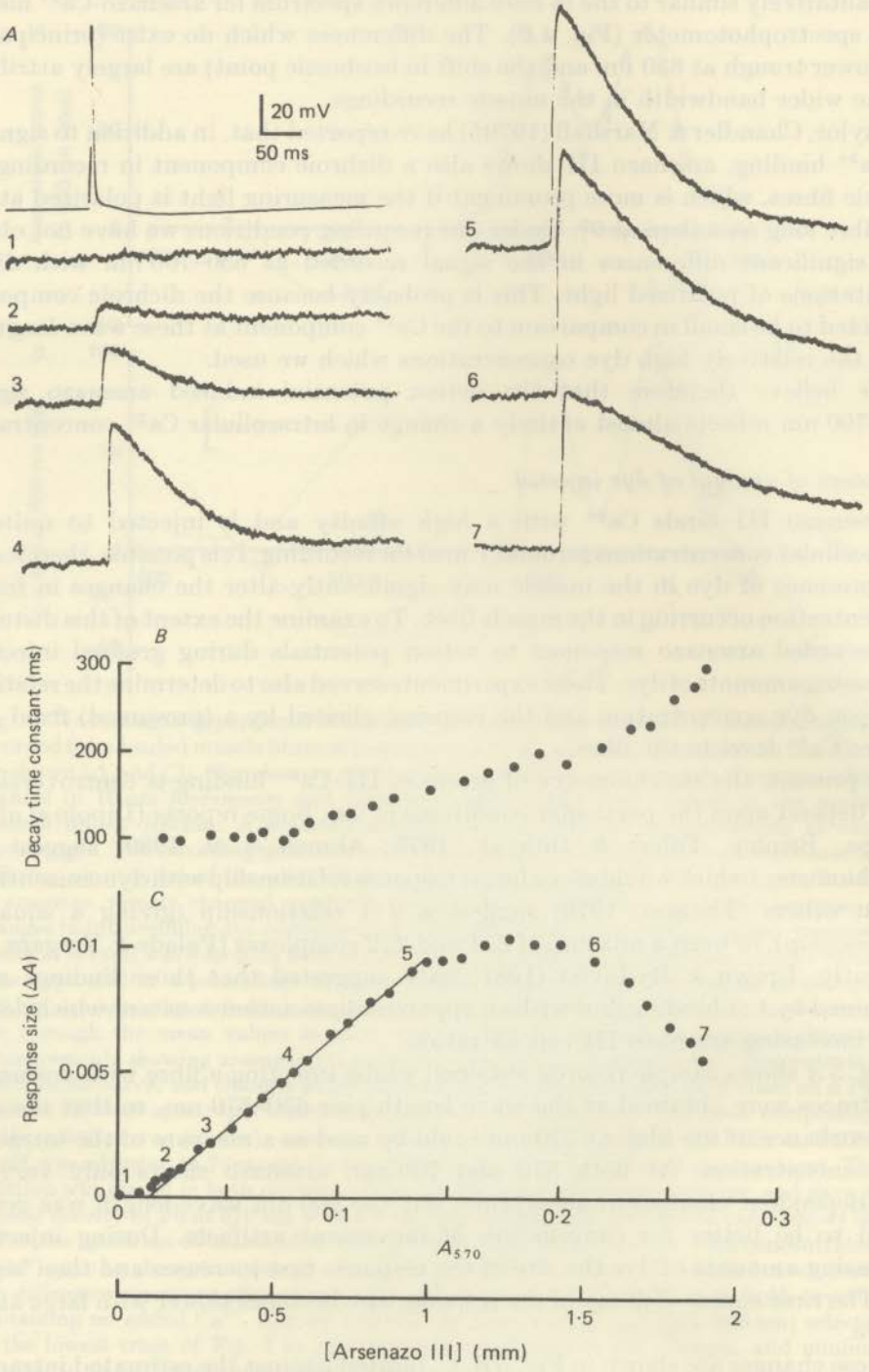

Fig. 5. For description see opposite.

1 mM the response reaches a maximum, and then begins to decline. The extrapolated linear relationship has a positive intercept on the dye concentration axis, indicating that at low concentrations (< 100 μM) the response is smaller than expected for the linear relationship. The decay time of the response (time to fall to 1/e of the peak) remains fairly constant for concentrations up to about 800 μM, but then begins to increase with increasing concentration.

The linear relation followed over a wide concentration range (100 to 1000 μM) suggests that under these conditions, one molecule of dye is involved in forming the dye–Ca^{2+} complex occurring during the peak of the optical signal. The origin of the deviation at low concentrations is not clear, and the decline in response size at high concentrations is also puzzling. If the dye were simply acting as a Ca^{2+} buffer, then it would be expected (assuming 1 : 1 stoichiometry) that the response should increase linearly with dye concentration at low levels – since a relatively constant free Ca^{2+} concentration change would be maintained by cytoplasmic Ca^{2+} buffers – but would curve off to a plateau at high levels where the dye became the major buffer and bound most of the total Ca^{2+} released.

The deviation from a linear dose–response relationship, and the slowing of response decay at higher dye concentrations both suggest that at arsenazo concentrations above about 1 mM the buffering power of the dye becomes sufficient to cause some disturbance of the free Ca^{2+} transients in the muscle. For these reasons, the results presented in this paper were obtained using moderate dye concentrations (O.D. at 570 nm of $< 0{\cdot}1$; corresponding to 700 μM arsenazo III for a typical 70 μM diameter fibre).

Changes in free and total Ca^{2+} during a twitch

Numerous *in vitro* calibrations are in agreement that the absorbance change of arsenazo III varies linearly with free Ca^{2+} concentration, for levels up to a moderate fraction of the dissociation constant (Scarpa, 1979; Gorman & Thomas, 1978; Miledi *et al.* 1977*a*; Thomas, 1979). A linear relationship has also been found to hold for ionophoretic injection of Ca^{2+} pulses into arsenazo-loaded muscle fibres (Miledi *et al.* 1981*b*) and, together with the observed linearity with dye concentration, simplifies attempts to calibrate the signals in terms of free Ca^{2+} concentration changes.

Fig. 5. Dependence of the arsenazo response to action potentials on the amount of dye injected into the fibre. *A*, traces show the light absorbance changes at 650–570 nm elicited from one fibre during injection of increasing amounts of dye. The upper left hand trace shows membrane potential, and the remaining traces show light transmission. The size and time course of the action potential did not change appreciably during the experiment. Numbers next to the traces correspond with the marked points in *B*, and indicate the effects of gradually increasing intracellular dye concentration over a period of about 15 min, starting from zero (trace 1). The ΔA calibration altered slightly from 0·0013 in trace 1 to 0·0015 in trace 7. *B*, plot of decay time constant of the arsenazo signal against amount of dye injected. Decay time was measured as time to fall to 1/e from the peak. The amount of dye injected was estimated from the increase in optical density at 570 nm. The calibration in terms of intracellular dye concentration assumes that the dye distributed evenly through the volume of the fibre covered by the measuring slit (fibre diameter 71 μm), and uses a molar extinction coefficient of $2{\cdot}6 \times 10^4$ for arsenazo under physiological conditions (see legend to Fig. 4). *C*, change in peak size of the arsenazo signal with amount of dye injected. Responses are expressed as ΔA.

Measurements were made of the peak response size following action potentials in nineteen fibres at a temperature of 9–11 °C. The dye concentration in the fibres was maintained on the linear portion of the concentration–response curve and the mean optical density of the injected dye at 570 nm (A_{570}) was $0{\cdot}086 \pm 0{\cdot}03$ (± 1 S.D.). To standardize for differences in fibre diameter and amounts of dye injected, measurements were expressed as $\Delta A/A_{570}$ (see Methods). This standardization assumes linearity of the response with respect to both dye and Ca^{2+} concentrations and neglects the non-zero intercept of the extrapolated linear dye concentration-dependence (Fig. 5*C*). Errors from this source would be small compared to other errors. The mean value of $\Delta A/A_{570}$ was $0{\cdot}079 \pm 0{\cdot}005$ (± 1 S.E.).

With saturating concentrations of Ca^{2+} the maximum value of $\Delta A/A_{570}$ for the test solution of Fig. 4*B* is 0·615. Hence, a free Ca^{2+} concentration equal to the apparent dissociation constant for arsenazo III will give a value of $\Delta A/A_{570} = 0{\cdot}307$, and at lower free Ca^{2+} concentrations the value of $\Delta A/A_{570}$ will vary in approximately direct proportion to the free Ca^{2+} concentration. Taking a value of 30 μM for the apparent dissociation constant for arsenazo III–Ca^{2+} under intracellular conditions (Brinley, Tiffert, Scarpa & Mullins, 1979; Ahmed *et al.* 1980) it follows therefore that the absorbance change during a twitch corresponds to a free Ca^{2+} concentration change of about 8 μM. This estimate should however be regarded as approximate in view of the assumptions above and because of the considerable influence of pH and Mg^{2+} concentration on the dye sensitivity.

We did not observe any appreciable change in peak size of the arsenazo response to an action potential with temperature. Mean values of $\Delta A/A_{570}$ at 6, 10 and 15 °C were respectively $0{\cdot}095 \pm 0{\cdot}0064$ (± 1 S.E. of mean; twelve fibres), $0{\cdot}079 \pm 0{\cdot}05$ (nineteen fibres) and $0{\cdot}081 \pm 0{\cdot}0082$ (six fibres).

From experiments similar to that illustrated in Fig. 5, it is possible to make a minimum estimate of the *total* amount of Ca^{2+} released during a twitch. At high intracellular concentrations of arsenazo III a large proportion of the released Ca^{2+} will bind to the dye and, from estimates of the absorbance change during the twitch ($\Delta A/A_{570}$) and the intracellular dye concentration, the amount of bound Ca^{2+} can be calculated. For example, in Fig. 5 the largest response was obtained at a dye concentration of about 1·5 mM, and gave a $\Delta A/A_{570}$ value of 0·05. The maximal value of $\Delta A/A_{570}$ is about 0·62 at saturating concentrations of Ca^{2+}, so the calculated amount of Ca^{2+} bound to the dye (assuming 1 : 1 stoichiometry) would have been $(0{\cdot}05/0{\cdot}62) \times 1{\cdot}5 = 120$ μM. From seven fibres a mean estimate of the total bound Ca^{2+} was obtained of 220 ± 20 μM (± 1 S.E.).

Decay phase of the arsenazo signal

The decay of the arsenazo response to an action potential follows a single exponential decline over most of the range. Fig. 6 shows examples of decay phases from several fibres at three temperatures, plotted on a semi-logarithmic scale. In healthy fibres it was generally found that the decay could be fitted well by an exponential from about 90 % of the peak value down to the limit of resolution at around 2 %. The first 10 % of the decline is often slower than expected from a single exponential. Some fibres also displayed a 'tail' on the decay, with a second, much slower, component for the decay to the base line from about 10–20 % of the peak.

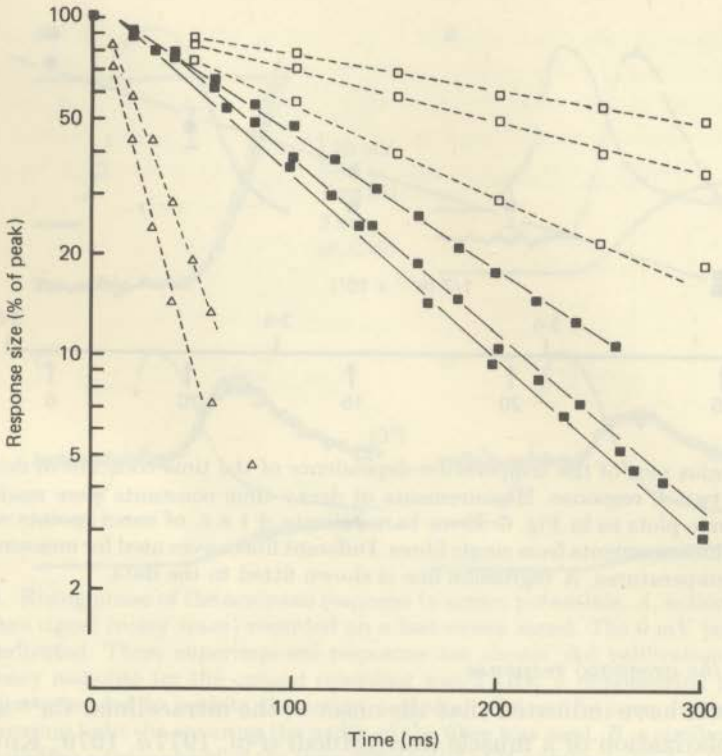

Fig. 6. Decay phase of the arsenazo response to the action potential. Measurements are shown of the decay phases from eight fibres at three temperatures (△, 25 °C; ■, 10 °C; □, 6 °C), plotted on a semi-logarithmic scale. Data has been normalized by expressing as a percentage of the peak value for each fibre. Zero time corresponds to the peak of the response. Straight lines are fitted by eye.

This 'tail' was most often seen in fibres which had a low resting potential, or which had been stimulated frequently. It was not usually detectable in fresh, healthy fibres. We feel therefore that the decay time course normally follows a single exponential, and that the 'tail' is present only in deteriorating fibres.

At a temperature of 10 °C a mean value of 71 ms (±11·5, S.E. of mean; eight fibres) was found for the decay time constant as measured from the slope of semi-logarithmic plots. Measurements were made from fibres showing no appreciable 'tail', but quite a large scatter was apparent between fibres, with a range of 58–112 ms. The relaxation time of the arsenazo–Ca^{2+} complex is about 3–4 ms (Scarpa, 1979; Palade & Vergara, 1981). Thus, the decay time of the arsenazo twitch response is unlikely to be limited by the kinetics of the dye reaction and instead presumably reflects the decline of free intracellular Ca^{2+}. This conclusion is strengthened by the wide scatter in decay times, which would not be expected if the dye reaction were the limiting factor.

The decay time constant showed a high temperature-dependence (Fig. 6). In Fig. 7 an Arrhenius plot is shown of decay times measured from different fibres over a range of temperatures (6–25 °C). The data can be fitted adequately by a straight line, with no evidence for any break points. The slope of the line corresponds to a Q_{10} of 2·4 over the range 10–20 °C.

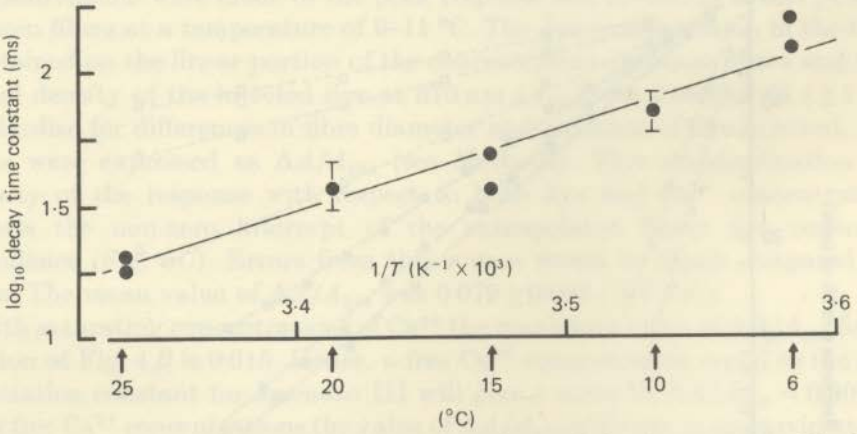

Fig. 7. Arrhenius plot of the temperature-dependence of the time constant of decline of the arsenazo twitch response. Measurements of decay time constants were made from semi-logarithmic plots as in Fig. 6. Error bars indicate ±1 S.E. of mean; points without error bars are measurements from single fibres. Different fibres were used for measurements at different temperatures. A regression line is shown fitted to the data.

Rising phase of the arsenazo response

Previous reports have indicated that the onset of the intracellular Ca^{2+} signal lags behind the depolarization of a muscle fibre (Miledi *et al.* 1977*a*, 1979; Kovács *et al.* 1979). The improved noise level of the present recording system allows this phenomenon to be seen more clearly following a single action potential (Fig. 8*A*). In this example, the optical trace remains flat for about 1·5 ms from the foot of the action potential (which corresponds to the threshold potential for Ca^{2+} release; Miledi *et al.* 1977*a*), and then begins to rise abruptly.

Since the rate of rise of the arsenazo signal is much faster than the rate of decay, it is possible to obtain a measure of the apparent rate of rise of free Ca^{2+} in the fibre by differentiating the optical signal. This is illustrated in Fig. 8*B*. The differentiated trace begins to rise with a latency of about 1·5 ms from the foot of the action potential, reaches peak about 3·5 ms later and has returned to the base line after about 10 ms.

One factor which might be expected to contribute to the latency of the optical signal is the time required for inward conduction of the action potential along the T-tubules (Gonzalez-Serratos, 1971; Adrian & Peachey, 1973; Nakajima & Gilai, 1980*b*). To test this possibility we recorded from a fibre using a narrow measuring light slit (about 15 μm wide and 300 μm long) which was positioned either along the edge or along the centre of the fibre. With the slit at the edge, signals would be detected only from regions of the fibre close to the surface membrane, and the inward transmission time to these regions would be short. On the other hand, the central slit would detect changes occurring throughout the whole fibre depth. Similar edge-centre optical recordings have previously been used to study radial propagation in the T-tubules using potential-sensitive dyes (Nakajima & Gilai, 1980*a*, *b*). Fig. 8*C*, *D* shows records obtained in this way from one fibre, using the differentiated optical trace as a monitor, since this shows up differences in latency more clearly. There is very little change

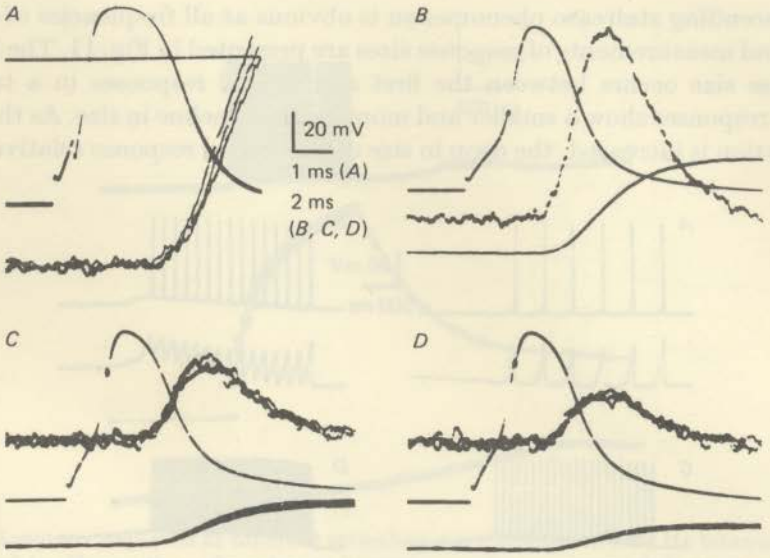

Fig. 8. Rising phase of the arsenazo response to action potentials. *A*, action potential and arsenazo signal (noisy trace) recorded on a fast sweep speed. The 0 mV potential level is also indicated. Three superimposed responses are shown. ΔA calibration is 0·0014 and frequency response for the optical recording was 2 kHz. A depolarizing pulse of 0·5 ms duration was used to initiate the action potential, and can be seen on the rising phase. A measuring light slit covering the width of the fibre was used. *B*, a similar record to (*A*), but including a trace showing the time differential of the arsenazo signal. Traces from top to bottom are: membrane potential, differential of the arsenazo signal, and arsenazo signal on a low gain. The record is of a single response, obtained from a different fibre to (*A*). Note the slower sweep speed. A simple RC circuit with a time constant of 200 μs was used to derive the differential trace. Frequency response for the optical recording was 1 kHz. Calibration for the optical trace is $\Delta A = 0{\cdot}015$; the differential trace was not calibrated. *C* and *D*, records obtained from the same fibre and in the same way as (*B*), except that the slit width was reduced to about 1/5 of the fibre diameter. In (*C*) the slit was positioned along the centre of the fibre, whilst in (*D*) it was positioned with the outer edge aligned along the edge of the fibre. Calibration is 0·04 ΔA for the arsenazo signal; differential trace uncalibrated. All other parameters as in (*B*).

detectable in latency or time course of the signal recorded with the slit at the edge or the centre of the fibre. Thus, radial propagation in the T-tubules appears to be responsible for only a very small part of the observed latency of the arsenazo signal.

Arsenazo responses during repetitive stimulation

Repetitive stimulation of a fibre elicits a series of arsenazo responses, which show progressive changes, and begin to summate above a certain frequency to give an increased peak level (Fig. 9). Even during prolonged tetanic stimulation the arsenazo record appears to be little contaminated by pH changes, although there may be some interference during the falling phase of the response after stimulation (Fig. 10). In agreement with Blinks *et al.* (1978), we observe two clear changes in the individual Ca^{2+} transients during a tetanus: (i) a gradual decline in the size of successive responses (the descending staircase), and (ii) a progressive slowing of decay of successive responses.

The descending staircase phenomenon is obvious at all frequencies of stimulation (Fig. 9), and measurements of response sizes are presented in Fig. 11. The largest drop in response size occurs between the first and second responses in a tetanus, and following responses show a smaller and more gradual decline in size. As the frequency of stimulation is increased, the drop in size of the second response relative to the first

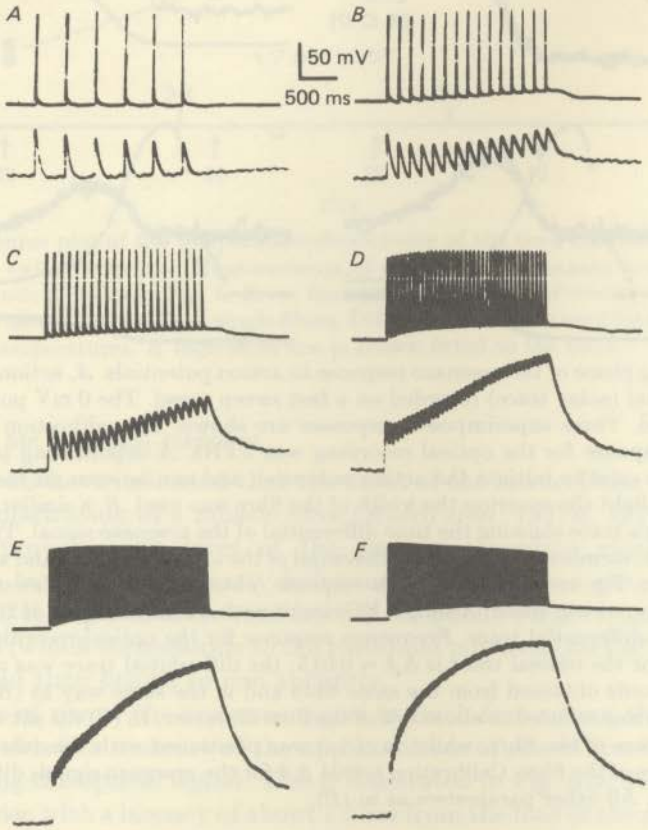

Fig. 9. Arsenazo responses to repetitive stimulation of various frequencies. In each record the top trace shows membrane potential and the lower trace light absorption at 650–700 nm. All records were obtained from one fibre, and intervals of 90 s were allowed between stimulation. Tetanus duration was 2 s, with frequencies of 3 Hz (*A*), 7·5 Hz (*B*), 10 Hz (*C*), 20 Hz (*D*), 30 Hz (*E*) and 40 Hz (*F*). ΔA calibration is 0·004. Optical signals were recorded with a 200 Hz frequency response.

becomes larger, but the subsequent decline during the following impulses becomes less steep. At all frequencies examined the gradual decline in response size after the second impulse appears to follow moderately well the same exponential function, which in Fig. 11 decays by 1/e after fifteen impulses.

A gradual slowing in decay time of the arsenazo response is apparent during repetitive stimulation. At low frequencies this can be seen during individual responses (Fig. 9*A*, *B*) and at high frequencies, where single responses are not discernable, it can be seen as a slowing of the decay following the tetanus (Fig. 9*C–F*). The decay

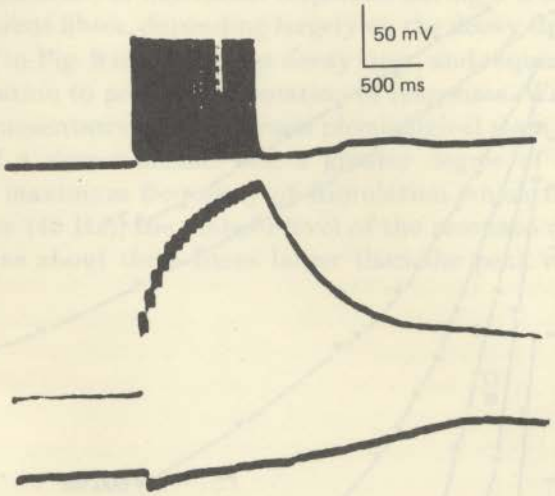

Fig. 10. Arsenazo responses at different recording wave-lengths to a 20 Hz tetanus for a duration of 1 s. Traces show, from top to bottom: membrane potential, light absorption at 650–700 nm and light absorption at 610–665 nm. The two optical records were obtained from two separate sweeps, with an interval of 90 s between stimulation. ΔA calibration is 0·0055 for the 610–665 nm trace and 0·005 for the 650–700 nm trace.

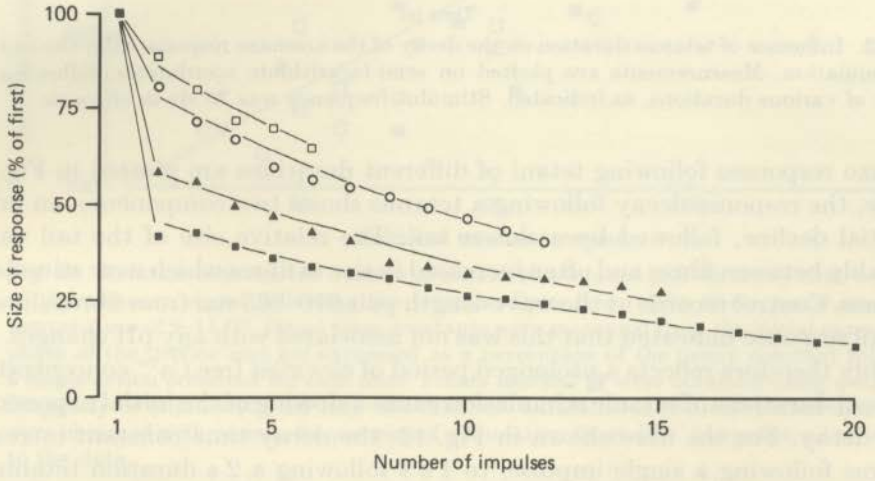

Fig. 11. Size of arsenazo transients elicited by successive action potentials during repetitive stimulation. The amplitude of each response was measured as the peak increase from the extrapolated decay phase of the preceding response, and is plotted as a percentage of the first response in each tetanus. The abscissa indicates the response number during the tetanus. Data are shown for four different frequencies of stimulation: □, 5 Hz; ○, 10 Hz; ▲, 20 Hz; ■, 40 Hz. All results from one fibre, which was different from that in Fig. 9. The curves drawn through the data points excluding the first value are all exponentials which decay by 1/e after fifteen impulses.

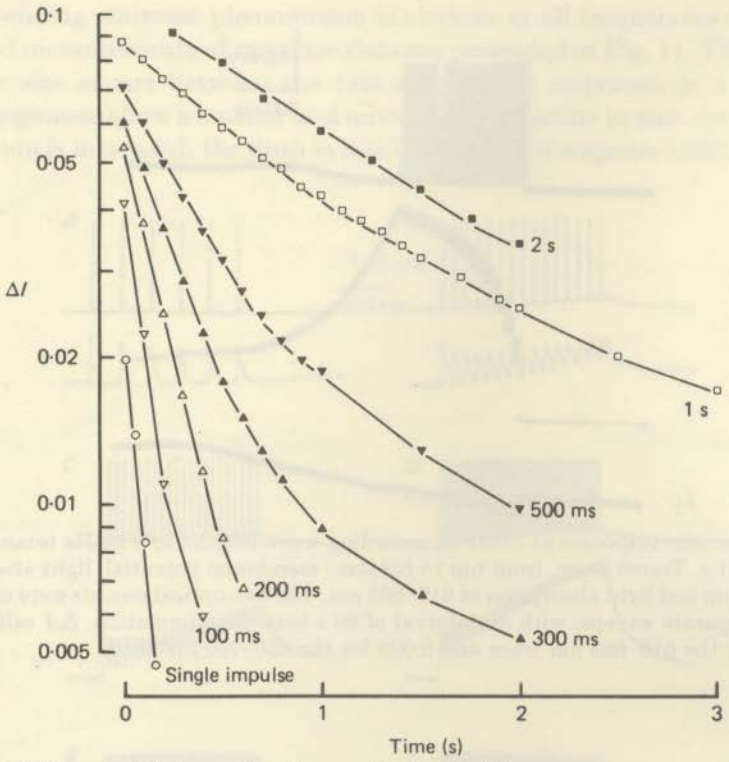

Fig. 12. Influence of tetanus duration on the decay of the arsenazo response after the end of stimulation. Measurements are plotted on semi-logarithmic coordinates, following tetani of various durations, as indicated. Stimulus frequency was 30 Hz in all cases.

of arsenazo responses following tetani of different durations are plotted in Fig. 12. Generally, the response decay following a tetanus shows two components, an initial exponential decline, followed by a slower tail. The relative size of the tail varied considerably between fibres and often increased in size in fibres which were stimulated many times. Control records at the wave-length pair 610–665 nm from fibres showing a large tail response indicated that this was not associated with any pH changes, and presumably therefore reflects a prolonged period of elevated free Ca^{2+} concentration.

Increased durations of tetanic stimulation cause a slowing of the initial exponential phase of decay. For the fibre shown in Fig. 12, the decay time constant increased from 95 ms following a single impulse, to 1·6 s following a 2 s duration tetanus at 30 Hz. The relationship between the number of stimuli in a tetanus and the decay time constant of the initial exponential decay is plotted from eight fibres in Fig. 13. An approximately linear relationship holds over the range of tetani examined (up to forty impulses), although there is a good deal of scatter. Measurements were made using both a constant frequency (30 Hz) and constant durations of tetanic stimulation. For both stimulus conditions the decay time constant appears to be related in the same way to the total number of impulses during the tetanus. Following a tetanus of forty impulses the decay time constant is slowed by a factor of about ten compared to the decay following a single impulse.

The degree of summation of individual responses during a tetanus varied considerably between different fibres, depending largely on the decay time of the responses. The fibre illustrated in Fig. 9 showed a fast decay time, and required a relatively high frequency of stimulation to produce summation of responses. We consider that this is probably most representative of the normal physiological state, since fibres in poor condition displayed a slower decline and a greater degree of summation at low frequencies. At the maximum frequency of stimulation which the action potential would reliably follow (40 Hz), the plateau level of the arsenazo response during the tetanus in Fig. 9 was about three times larger than the peak response to a single impulse.

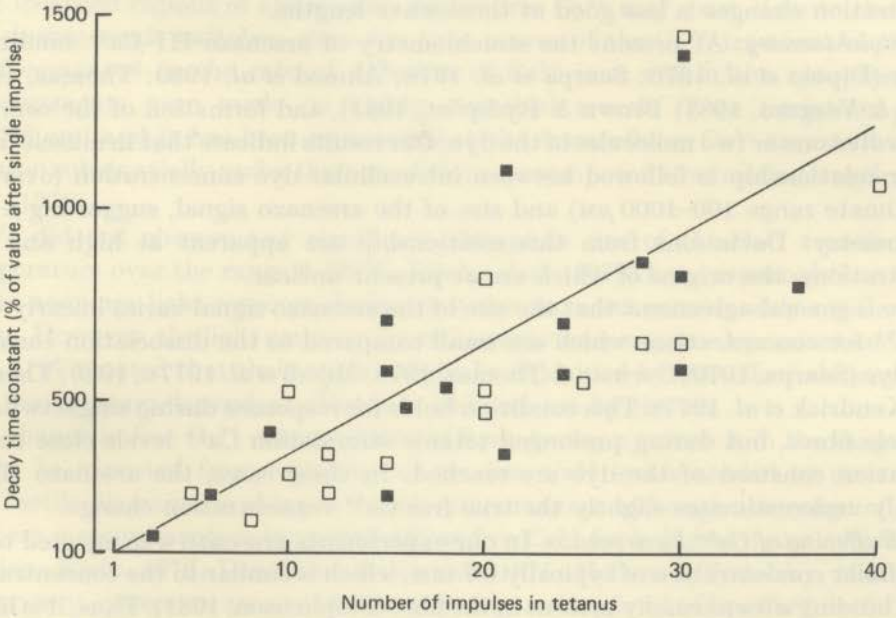

Fig. 13. Relation between the number of stimuli in a tetanus and the decay time constant of the arsenazo signal following the tetanus. Data from eight fibres are shown, at a temperature of 9–11 °C. Decay time constants were measured from the initial exponential phase of the decline and are expressed as a percentage of the decay constant following a single action potential for each fibre. Points marked ■ were obtained using a constant frequency of stimulation (30 Hz), and different durations of tetanus. Points marked □ were obtained with various frequencies of stimulation (3–40 Hz). A regression line is fitted to the data.

DISCUSSION

Use of arsenazo in muscle fibres

In several respects arsenazo III provides an excellent technique for studying Ca^{2+} transients in muscle fibres: it has good sensitivity, fast response time, an approximately linear response with free Ca^{2+} concentration, and is easy to use in practice. Some problems exist, however, of which the most important are probably: (i) specificity of the dye response, (ii) stoichiometry of dye–Ca^{2+} binding and linearity of the

response, and (iii) binding of significant amounts of muscle Ca^{2+} to the dye. In general we feel that these problems are not serious for many purposes.

(*i*) *Dye specificity.* Arsenazo III is sensitive to many ions, of which only Ca^{2+}, Mg^{2+} and H^+ are likely to be important in intracellular recordings. The change in absorption spectrum due to Mg^{2+} and H^+ is different from that due to Ca^{2+}, and by using differential recording at different wave-lengths we have been able to show that the arsenazo signals recorded at 650–700 nm during twitches and tetani reflect changes in Ca^{2+}, with only very small contamination from Mg^{2+} or pH changes. It is also likely that earlier measurements (Miledi *et al.* 1977*a*, 1980, 1981*b*; Miledi & Parker, 1981) made at the wave-length pair 602–532 nm were not significantly contaminated by interference from other ions, although rejection of pH and Mg^{2+} concentration changes is less good at these wave-lengths.

(*ii*) *Stoichiometry.* At present the stoichiometry of arsenazo III–Ca^{2+} binding is unclear (Dipolo *et al.* 1976; Scarpa *et al.* 1978; Ahmed *et al.* 1980; Thomas, 1979; Palade & Vergara, 1981; Brown & Rydqvist, 1981), and formation of the complex may involve one or two molecules of the dye. Our results indicate that in muscle fibres a linear relationship is followed between intracellular dye concentration (over the approximate range 100–1000 μM) and size of the arsenazo signal, suggesting a 1 : 1 stoichiometry. Deviations from this relationship are apparent at high and low concentrations, the origins of which are at present unclear.

There is general agreement that the size of the arsenazo signal varies linearly with free Ca^{2+} for concentrations which are small compared to the dissociation constant of the dye (Scarpa, 1979; Gorman & Thomas, 1978; Miledi *et al.* 1977*a*, 1980; Thomas, 1979; Kendrick *et al.* 1977). This condition holds for responses during single twitches in muscle fibres, but during prolonged tetanic stimulation Ca^{2+} levels close to the dissociation constant of the dye are reached. In these cases, the arsenazo signal probably underestimates slightly the true free Ca^{2+} concentration change.

(*iii*) *Buffering of Ca^{2+} by arsenazo.* In our experiments arsenazo was injected to an intracellular concentration of typically 0·7 mM, which is similar to the concentration of Ca^{2+} binding sites normally present in the fibre (Stephenson, 1981). Thus, it is likely that the presence of the dye may affect Ca^{2+} movements in the fibre. A slowing in decline of the arsenazo signal is in fact seen at higher dye concentrations, which may be due to slower uptake by the s.r. of Ca^{2+} ions bound to arsenazo. However, the observed change in decay time going from low dye concentration (which will presumably have only minimal effects on Ca^{2+} movements) to a concentration of about 0·7 mM is only a factor of about 1·2. Measurements of absolute values of decay times following twitches and tetani may therefore be slightly over-estimated, but relative changes in decay time under different conditions are probably reliable. For the purpose of looking at the amounts of Ca^{2+} released by the s.r. under different conditions, Ca^{2+} buffering by the dye is probably not important.

This problem of Ca^{2+} buffering will be common to all intracellular Ca^{2+} monitoring techniques using indicator dyes. The recorded optical signal is proportional to the amount of Ca^{2+} bound to the dye and, since arsenazo III gives a large absorbance change on binding, it is unlikely that any other dyes could improve appreciably on this aspect. Further improvements in the optical recording system would be required

to allow Ca^{2+} buffering to be minimised by using smaller intracellular concentrations (or dyes with a lower affinity), whilst maintaining the signal to noise ratio.

Amount of Ca^{2+} released during twitches and tetani

Our estimate for the peak increase in cytoplasmic free Ca^{2+} concentration following an action potential is about 8 μM. The estimate however depends upon the value of the apparent dissociation constant of arsenazo III for Ca^{2+} (assumed to be 30 μM). Since the details of arsenazo binding with Ca^{2+} are at present controversial, some revision may be necessary if better estimates of the dissociation constant become available. The arsenazo technique gives a measure of the average free Ca^{2+} level throughout the fibre volume covered by the measuring light. It is possible, therefore, that localized regions of appreciably higher free Ca^{2+} may exist close to the release site during single twitches, since the time course of the Ca^{2+} transient is probably brief compared to the rate of diffusion of Ca^{2+} ions within the cytoplasm. Our measurements were made on highly stretched muscle fibres (striation spacing 3·6–3·8 μm) and it has been reported that the intracellular Ca^{2+} transient may be reduced substantially under these conditions, as compared to at resting length (Blinks *et al.* 1978).

We did not observe any significant change in size of the Ca^{2+} transient with temperature over the range 6–25 °C. Blinks *et al.* (1978) have reported that the size of the aequorin light response during twitches increases considerably over a similar range. However, the light emission by aequorin is temperature-dependent and Blinks *et al.* (1978) noted that their results might be explained by this effect alone, without any temperature-dependent change in Ca^{2+} release by the s.r.

A change in free Ca^{2+} concentration of 8 μM during a twitch is in the range which might be expected from estimates of the free Ca^{2+} levels needed to activate the contractile filaments in skinned muscle preparations, although the range of scatter in these measurements is large (0·5–10 μM-free Ca^{2+} for half maximum activation of tension; Endo, 1976; Donaldson & Kerrick, 1975; Hellam & Podolsky, 1969). With our present system we are unable to resolve the resting level of free Ca^{2+} in the muscle fibre.

From measurements of the amount of Ca^{2+} bound to arsenazo in heavily loaded fibres, it is possible to make a minimum estimate of the total amount of Ca^{2+} released during a twitch. Our estimate is about 220 μM-Ca^{2+} per litre fibre water, which is somewhat higher than the estimate of 100 μM made by Baylor *et al.* (1979*a*), using similar methods. The total amount of Ca^{2+} contained in the s.r. corresponds to about 700 μM (Endo, 1977; Gilbert & Fenn, 1957); thus a single action potential appears to release about one quarter of the total Ca^{2+} in the s.r. During a tetanus we find that the arsenazo signal can rise to a level three or four times higher than during a single twitch, and the true Ca^{2+} concentration may be greater than this due to non-linearity of the arsenazo signal at high Ca^{2+} levels. Under these conditions it seems that nearly all of the muscle Ca^{2+} must be redistributed from the s.r. to the cytoplasm.

Even during a long tetanus, the released Ca^{2+} appears to be well buffered by Ca^{2+} binding systems in the muscle cytoplasm. If this were not the case, an abrupt rise

in free Ca^{2+} would be expected when the cell buffering systems became saturated. This has not been seen either in our results, or in recordings with aequorin (Blinks *et al.* 1978). Estimates of the total Ca^{2+} binding capacity in the muscle cytoplasm (principally troponin, myosin and parvalbumins) are in the range 0·7 to 1·4 mM (Stephenson, 1981), so that during tetani the binding systems are probably at least half-saturated.

Rising phase of the arsenazo signal

A latency of about 1·5 ms (at 10 °C) is seen between the rising phase of the action potential and the onset of the arsenazo signal. One possible explanation for this delay is that it arises from the kinetics of the dye reaction with Ca^{2+}. Estimates of the response time of arsenazo III range from 200 μs (Brown *et al.* 1975) to 1–2 ms (Palade & Vergara, 1981; Scarpa, 1979), although there may be more than one time constant involved (Palade & Vergara, 1981). For several reasons it seems unlikely that the observed latency can arise from the reaction time of the dye: (i) slow kinetics would be expected to give a slowly rising signal, rather than an abrupt transition from a flat base line to a steep rate of rise, (ii) the delay is affected by factors which would not be expected to affect the dye reaction time (Miledi *et al.* 1979), and (iii) a closely similar delay has been reported using antipyrylazo III (Palade & Vergara, 1981; Kovács *et al.* 1979), a dye which has a faster relaxation time (Palade & Vergara, 1981; Scarpa, 1979). The observed latency of the arsenazo signal therefore probably results from the excitation–contraction coupling process in the muscle.

One factor which may be expected to contribute to the delay is the conduction time of the action potential inward along the T-tubules. Conduction to the centre of a 70 μm diameter fibre would take about 0·5 ms at 20 °C (Gonzalez-Serratos, 1971; Nakajima & Gilai, 1980*b*), but any contribution to the latency of the arsenazo signal would be smaller than this, since the initial signal will be recorded from peripheral regions of the fibre. In agreement, we find little difference in the latency when arsenazo signals are recorded with a narrow light slit positioned either at the edge of a fibre (so as to record only from peripheral regions), or at the middle of the fibre (to record mainly from deeper parts of the fibre).

Following the initial latency, the arsenazo signal reaches a peak after a further 10 ms. The differential of the signal is shown in Fig. 8, and it is tempting to suppose that this reflects the rate of increase in free Ca^{2+} concentration, and hence the time course of Ca^{2+} permeability change of the s.r. membrane. However, the time course of the arsenazo signal may be distorted by at least two factors. One is the uncertain kinetics of the dye response, and another involves the diffusion time of Ca^{2+} ions in the muscle cytoplasm. Although arsenazo generally gives a measure of the average free Ca^{2+} level, this relation will break down if localized concentrations become sufficiently high to saturate the dye, and could give a signal which continues to rise after Ca^{2+} release has stopped (Miledi & Parker, 1981). Whatever the case, the differential trace in Fig. 8 sets a maximum time course for the release of Ca^{2+} from the s.r., and it seems that the majority of Ca^{2+} release must occur after the membrane potential has returned to a level more negative than that required to activate excitation–contraction coupling.

Decay of the arsenazo signal

The decay of the arsenazo response to an action potential largely follows a single exponential, which has a time constant of about 70 ms at 10 °C. This decay time is appreciably slower than estimates of the relaxation time of the dye (Scarpa, 1979; Palade & Vergara, 1981), so that it presumably gives a true indication of the fall in free Ca^{2+} concentration of the fibre. Several mechanisms might be expected to contribute to the lowering of the free Ca^{2+} level: for example, active uptake by the s.r. and mitochondria, and binding to troponin, myosin and parvalbumins. However, the observation that the decay follows a single exponential component suggests that it is principally determined by only one mechanism, and additional observations suggest that this mechanism is probably the active uptake of Ca^{2+} by the s.r. In agreement with F. Eusebi, R. Miledi & T. Takahashi (unpublished observations), we find that the temperature-dependence of the decay time constant is high, with a Q_{10} of about 2·4. This value is similar to measurements of the Q_{10} of active uptake of Ca^{2+} by isolated s.r. vesicles (Q_{10} = 2·3–3; Weber, Herz & Reiss, 1966; Ogawa, 1970), and is more consistent with an active metabolic process than a simple binding reaction. Active uptake of Ca^{2+} by the mitochondria is thought not to play any important role in muscle (Ebashi, 1976).

Following repetitive stimulation, the decline of the arsenazo signal is slower than following a single twitch, and the decay time constant appears to increase linearly with the number of impulses during the tetanus. It is difficult to envisage how this could be explained if the decline is due to Ca^{2+} binding, and instead it seems most likely that the rate of uptake of Ca^{2+} by the s.r. becomes slowed following tetanic stimulation.

Ca^{2+} transients during repetitive stimulation

When a fibre is stimulated to give a train of action potentials, a series of arsenazo signals are recorded which summate, and at frequencies above about 10 Hz (at 10 °C) give rise to a higher level than reached at the peak of a single response. As regards activation of the contractile filaments, it seems that the peak levels of free Ca^{2+} during a twitch are sufficient to saturate tension development (Blinks *et al.* 1978). Instead, the proportion of time for which the Ca^{2+} concentration is above the level for contractile activation may be the important factor. In this respect it is interesting that the decay times of the Ca^{2+} transients become proportionally slower with increasing number of action potentials in a tetanus.

During a tetanus, the amount of Ca^{2+} released by successive action potentials decreases, but this effect is largely offset by the slowing of the rate of decline, so that the level of free Ca^{2+} remains steady, or increases, during stimulation at most frequencies. Similar findings have been observed previously using aequorin (Blinks *et al.* 1978), and in a following paper we will discuss possible mechanisms for the decrease in release during tetani. At present, it may be noted that these phenomena, taken together, provide a means by which the cytoplasmic Ca^{2+} level may be maintained during a tetanus with a much reduced metabolic expenditure on Ca^{2+} release and re-uptake. A similar mechanism has previously been postulated for slow muscle fibres (Miledi *et al.* 1981).

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