USE OF ARSENAZO III FOR RECORDING CALCIUM TRANSIENTS IN FROG SKELETAL MUSCLE FIBRES

I. PARKER

Biophysics Department, University College, Gower Street, London WC1E 6BT

SUMMARY

Arsenazo III has been used to study calcium transients in twitch and slow frog muscle fibres under current and voltage clamp conditions, as well as at the neuromuscular junction. The advantages and disadvantages of this technique and the problem of *in vivo* calibration of the absorbance signal are discussed.

INTRODUCTION

The key role of Ca^{2+} ions released from the sarcoplasmic reticulum in the activation of contraction in vertebrate skeletal muscle is now well established (for recent reviews see Ebashi, 1976; Endo, 1977). The first direct indications of free Ca^{2+} transients occurring during contraction were made using the indicator dye murexide (Jöbsis & O'Connor, 1966), but this was very insensitive, and most subsequent work has used aequorin (see, for example, Blinks, Rüdel & Taylor, 1978).

This article describes techniques for using the metallochromic indicator dye arsenazo III for recording changes in intracellular free Ca^{2+} concentration in muscle fibres. Free Ca^{2+} transients can also be recorded during contraction of twitch and slow muscle fibres (Miledi, Parker & Schalow, 1977b, c), as well as during Ca^{2+} entry across the post-junctional membrane (Miledi, Parker & Schalow, 1977a), and these results are also discussed here.

TECHNIQUES

Preparation of Muscle

For recording free Ca^{2+} transients in twitch muscle fibres the cutaneous pectoris muscle of *Rana temporaria* was used, (Blioch, Glagoleva, Liberman & Nenashev, 1968). This muscle is a thin sheet only a few fibres thick, and is ideal for optical recording of transmitted light since there is little light absorption or scatter.

However, movement artefacts during contraction of the muscle interfere seriously with the optical recording, and the muscle was stretched to reduce

filament overlap, and this minimised contraction. Threads were attached to each end of the muscle, and metal hooks were inserted into the connective tissue along the sides of the muscle. Stimuli were then applied through external wire electrodes, and the threads and hooks tightened until no twitching was visible. It was impossible to eliminate contractions in all fibres in the muscle, but usually regions could be found where the striation spacing was about 3.6 μm, and where stimulation of single fibres did not produce significant movement artefacts.

For studies on slow muscle fibres, the pyriformis muscle of *Rana temporaria* is a convenient preparation. The muscle is, however, too thick for optical recording, and it was necessary to cut away the lower part, leaving only a thin sheet of fibres. This was then mounted and stretched in the same way as for the twitch muscle fibres. Each preparation contained several slow fibres, which could be identified by their slow electrotonic time constant after penetration by microelectrodes.

Electrical Recording and Dye Injection

Fibres were penetrated with two micro-pipettes, for voltage recording and dye injection. The pipette tips were separated by about 200 μm, so that the light spot for optical recording could be positioned between them. The arsenazo III pipette was connected to a high voltage (±100 V) constant current generator, which facilitated the passing of steady iontophoretic currents for dye injection. This pipette also served as a current-passing electrode either for stimulating the fibre with depolarising pulses, or for voltage clamping.

Arsenazo III pipettes were filled with an aqueous solution of about 1 mM arsenazo III (practical grade, Sigma Chemical Co.), together with 10 mM $KHCO_3$, at pH 7. The bicarbonate increased the solubility of the dye (Savvin, 1966), and helped prevent blocking of the pipette tips. The injection was further improved by bevelling the pipette tips (as described by Chang, 1975) to a diameter of about 1 μm. The arsenazo III was used without any purification, and gave satisfactory results, although it is known to be contaminated with Ca^{2+} and other dyes (Kendrick, 1976). Calibrations were made with the same batch as used for injection.

Steady pipette negative currents of 10-50 nA were used to inject arsenazo III into muscle fibres over a period of about 10 min. The progress of injection was monitored by an increase in absorbance of the fibre at 532 nm.

Optical Recording

The arsenazo III absorbance change which occurs during muscle twitches is quite fast, and requires a bandwidth of at least 100 Hz in the recording system to avoid distortion of the signal. The optical system was designed for

maximum sensitivity giving an acceptable signal-to-noise ratio with this bandwidth.

Simultaneous measurements of absorbance were made at wavelengths of 532 and 602 nm, where arsenazo III displays respectively a maximum decrease and increase in the presence of Ca^{2+}. By subtracting these two signals a recording could be obtained in which artefacts tending to affect both channels equally (*e.g.* movements or changes in light scattering of the muscle) were substantially cancelled out, whilst changes in absorbance of the dye summated. Other wavelength pairs (*e.g.* 675-685 nm: Scarpa, Tiffert & Brinley 1977) would give greater immunity to possible changes in Mg^{2+} concentration or pH, but have a lower sensitivity.

Photomultipliers were used for light measurement, in preference to photodiodes, because of their inherently higher stability and lower noise level. The noise level of the recordings was principally determined by statistical variations in the number of photons reaching the photomultipliers. The largest muscle fibres present were therefore selected, to allow the use of a large diameter (80-150 μm) measuring light spot.

The optical system is shown in Fig. 1, and was based upon a modified Watson 'Bactil' microscope. Light from a 50W lamp was focused through the glass base of the chamber onto a surface fibre of the muscle. An iris diaphragm allowed the diameter of the light spot to be adjusted to the diameter of the muscle fibre. An objective lens, fitted with a 'dipping cap' to permit immersion in the bathing fluid, collected the transmitted light which was diverted by a system of beam-splitting mirrors onto two photomultipliers (EMI 9524B). Interference filters (Balzers: half-peak bandwidths 12 nm) transmitting wavelengths of 532 and 602 nm were placed in front of the photomultipliers.

Virtual earth circuits were used to convert the anode currents of the photomultipliers into voltage signals, which were then electronically subtracted. Before injecting dye, the gains of the two photomultiplier channels were adjusted to give equal signals for the transmitted light, and the amount of dye injected was then monitored by the fall in transmitted light at 532 nm.

Calibration of Optical Records

A calibration of the optical records in terms of changes in free Ca^{2+} concentration was made by replacing the muscle with a glass capillary of internal diameter 90 μm, and passing through this solutions of arsenazo III containing known free Ca^{2+} concentrations. The composition of the calibrating solution resembled the ionic composition of the myoplasm, and contained (mM):

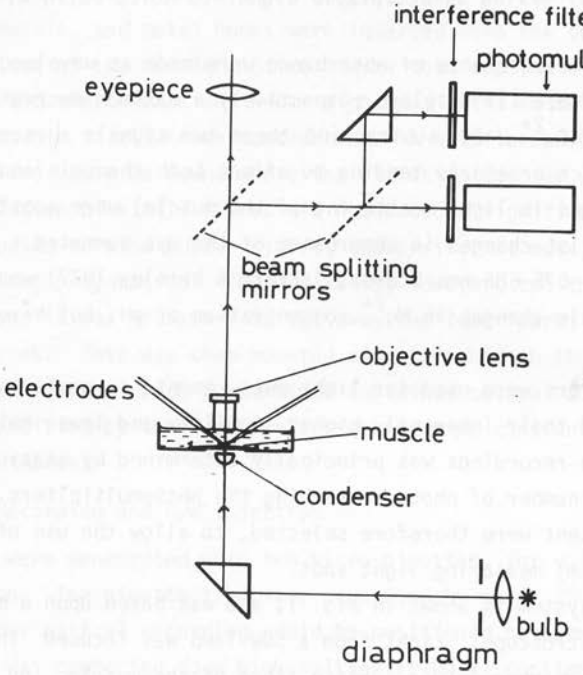

Fig.1: Optical system for dual wavelength recording of absorbance changes in muscle fibres. The light source is a 50 W quartz halogen lamp with well-stabilised power supply. A high-aperture condenser system focuses light onto a surface muscle fibre; the spot diameter is set by a diaphragm. Light transmitted through the fibre is collected by a x 10 microscope objective immersed in the bathing fluid, and split equally by a beam-splitting mirror. One beam is used to view the preparation through an eyepiece; whilst the other is split again and directed onto two photomultipliers.

100 KCl, 2 $MgCl_2$, 0.2 arsenazo III, in 4 mM phosphate buffer, pH 7.0, also 1 mM EGTA was added to the solution to buffer free Ca^{2+} levels below 1 μM, and the arsenazo III was used as a buffer for higher Ca^{2+} concentrations. The arsenazo III concentration used gave a decrease in light transmission of 5% at 532 nm in the capillary, which was approximately the same as in experiments with muscle fibres of the same diameter.

In order to standardise for differences in muscle fibre diameters, and different amounts of dye injected, the photomultiplier records were expressed as the change in absorbance of the dye at the wavelength pair 532-602 nm due to Ca^{2+}, divided by the absorbance of the dye at 532 nm in Ca^{2+} free solution; *i.e.* standardised change in absorbance due to Ca^{2+} $\Delta A_S = (\Delta A_{532} - \Delta A_{602})/A_{532}$. This standardization assumes that the light response for a given change in free

Ca^{2+} concentration varies linearly with dye concentration; that is to say, that the binding between Ca^{2+} and arsenazo III is in a 1:1 relation (Michaylova & Ilkova, 1971; Savvin, 1966). Recently, however, Gorman & Thomas (1978) report finding a square-law relation between light response and dye concentration. The results reported here are not in agreement with this finding, but instead indicate that under the conditions used in the muscle experiments there is a linear relation between dye concentration and light response (Miledi, Parker & Schalow, unpublished data).

Figure 2 shows results of an *in vitro* calibration, using a spectrophotometer. A constant free Ca^{2+} concentration was maintained in the calibration solution by an EGTA buffer, and the change in absorbance at the wavelength pair 532-602 nm was measured for different arsenazo III concentrations, using a reference solution of the same composition, but with virtually no free Ca^{2+}. The absorbance change was found to be a linear function of dye concentration up to about 100 μM, and the relation appeared to 'round off' at higher concentrations.

Similar results were obtained from *in vivo* experiments, where the size of the light response elicited during single twitches was recorded during injection of the dye (Fig. 3). A measure of the amount of dye injected was obtained from the increase in absorbance of the fibre at 532 nm. From calibrations with the glass capillary it was estimated that an increase in absorbance of 0.05 corresponded to an arsenazo III concentration of approximately 200 μM. It was expected that the amount of free Ca^{2+} released during each twitch would be constant. The size of the light response was a linear function of dye concentration during the initial stages of the injection but reached a plateau, and then began to fall as the injection was continued. The reason for the non-linearity at higher dye concentrations is not clear, but appears to be a property of the dye, since it was seen in both *in vivo* and *in vitro* experiments, and was associated with a broadening of the absorbance peak of the dye under Ca^{2+}-free conditions.

The initial linear relation between size of the response and the amount of arsenazo III injected into a muscle fibre, provides evidence that over this concentration range the injected dye is not significantly buffering the internal Ca^{2+}. Quantitative measurements of the size of light responses were always made with the dye concentration within the linear range. In other experiments sufficient dye was injected to reach the plateau range, so that small changes in dye concentration in the fibre during an experiment would have little effect on the light response size.

For absorbance changes up to ΔA_S values of about 0.3, a linear relation was found between ΔA_S and free Ca^{2+} concentration, with a slope of 20 μM per unit ΔA_S.

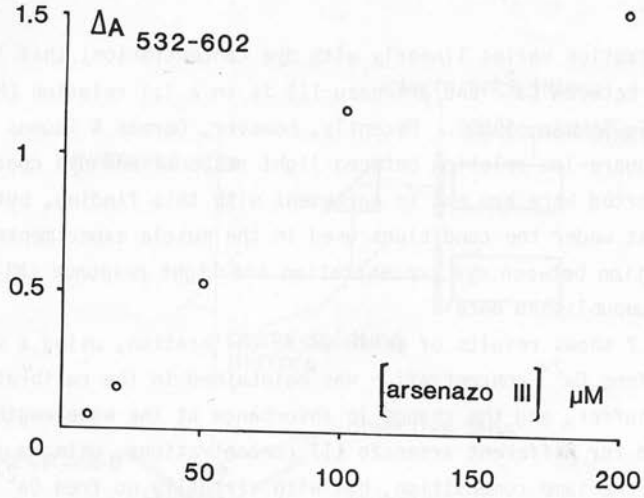

Fig. 2: The effect of dye concentration on the Ca/arsenazo III absorbance change in vitro. Abscissa; arsenazo III concentration in μM. Ordinate; absorbance (Δ_A) of the test solution at the wavelength pair 532-602 nm, measured against an identical solution with virtually zero free Ca^{2+}. Test solution contained (mM): 110 KCl, 10 EGTA, 6 $CaCl_2$, and 10 phosphate buffer at pH 7.25. $CaCl_2$ was omitted from the reference solution. Readings were made with a dual beam spectrophotometer, with an optical path length of 1 cm. Dye concentration was calculated by weight of dye.

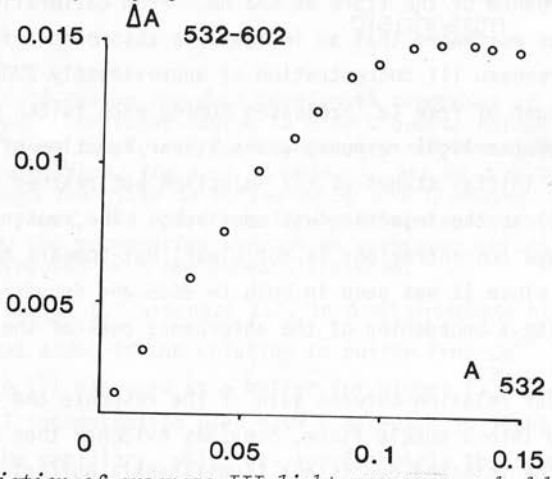

Fig. 3: Variation of arsenazo III light response evoked by action potentials during injection of a fibre with dye. Abscissa; fractional increase in absorbance at 532 nm resulting from injection of dye. Ordinate; peak change in absorbance at the wavelength pair 532-602 nm, occurring during single action potentials. Temp. 8°C.

At higher free Ca^{2+} concentrations the dye response is no longer linear, resulting in a limiting value for ΔA_S of about 0.9 (Miledi et al., 1977b).

RESPONSE OF ARSENAZO III IN SINGLE MUSCLE FIBRES

Calcium Injections into Muscle Fibres

Iontophoretic injection of free Ca^{2+} into arsenazo III-loaded muscle fibres produced an increase in absorbance at 602 nm, and a smaller decrease at 532 nm. Fig. 4 illustrates the light response at the wavelength pair 532-602 nm resulting from a 0.5 sec duration Ca^{2+} pulse, and compares this with the response elicited by an action potential in the fibre.

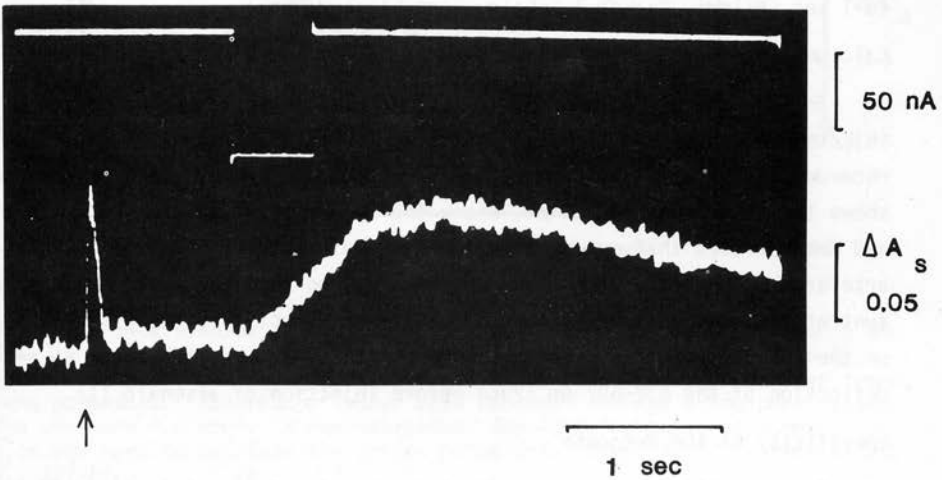

Fig.4: Absorbance records at 532-602 nm from a fibre injected with arsenazo III. Responses to an action potential (arrow), and to an iontophoretic pulse of Ca^{2+} (0.4 M-$CaCl_2$) injected into the fibre from a pipette. Upper trace, iontophoretic current through the Ca^{2+} pipette; lower trace, absorbance. A measuring light spot (ca. 80 µm dia.) was used, with the Ca^{2+} pipette tip in the centre. A 2 msec depolarising pulse was passed through the pipette containing the dye and initiated the action potential. The Ca^{2+} pulse depolarised the fibre from -90 to -55 mV; but the same depolarisation applied through the pipette containing the dye gave no light response. A backing current of 9 nA was applied to the Ca^{2+} pipette. Recording time constant on the optical trace was 10 msec. Temp. 8°C.

Both the time to peak and the decay of the response to the Ca^{2+} pulse were very much slower than the action potential response. This slow time course probably results from the slow diffusion of Ca^{2+} within the muscle fibre. In the case of the action potential, Ca^{2+} ions are released from the sarcoplasmic reticulum at sites distributed throughout the fibre at spacings of a few microns. Homogeneous mixing of Ca^{2+} with arsenazo III will therefore occur rapidly. Iontophoretic injection of Ca^{2+} on the other hand will give rise to a very

localised region of high Ca^{2+} concentration around the pipette tip. The resulting light response will be non-linear if the local Ca^{2+} concentration is sufficiently high to saturate the dye, and an increase in response would be expected as Ca^{2+} ions diffuse out into a larger volume of the fibre. The slow decline of the response may be due to a local saturation of the Ca^{2+} uptake and binding systems of the fibre.

A linear relation was observed between the total charge injected through the Ca^{2+} pipette (up to 50 n coulomb), and the peak size of the light response. This relation was independent of the length of the Ca^{2+} pulse for durations up to 1 sec (Miledi, Parker & Schalow, unpublished data).

Calcium Transients during Twitches

The change in absorbance which accompanies an action potential in a fibre injected with arsenazo III is illustrated in Fig. 5, a large change being recorded at the wavelength pair 532-602 nm (middle trace). The upper trace shows the absorbance at 532 nm, where the dye is relatively insensitive to Ca^{2+}, and demonstrates that movement of the fibre did not give rise to any serious artefacts. The deflection below the baseline on this trace was produced by contraction of the fibre, but would have been almost completely cancelled out on the 532-602 nm trace. In correctly stretched fibres, there was no detectable deflection of the 532-602 nm trace before injection of arsenazo III.

Specificity of the Response

Arsenazo III is not a specific indicator for Ca^{2+} but is also affected by many other ions, and by changes in pH (Savvin, 1966; Michaylova & Ilkova, 1971; Kendrick, Ratzlaff & Blaustein, 1977; Gorman & Thomas, 1978). Of these factors, only changes in free Mg^{2+} and pH are likely to be of biological significance.

The sensitivity of arsenazo III for Mg^{2+} is low, the affinity for this ion being about 100- to 200-fold less than for Ca^{2+}, (Kendrick et al., 1977) and an increase in Mg^{2+} concentration of over 1 mM would have been required to produce the absorbance change shown. Abolition of the light response by injection of EGTA into fibres (Suarez Kurtz & Parker, 1977) provides further evidence that the absorbance changes result from changes in Ca^{2+} concentration, and not Mg^{2+}, since this compound binds Ca^{2+} over 10^5 times more effectively than Mg^{2+}.

Changes in intracellular pH during contraction could give rise to absorbance changes of the dye, since the intracellular Mg^{2+} concentration is about 3 mM (Cohen & Tyler Burt, 1977), and the absorbance of the Mg-dye complex is quite sensitive to pH over the physiological range (Kendrick et al., 1977). However, it has been demonstrated that the intracellular pH of muscle fibres

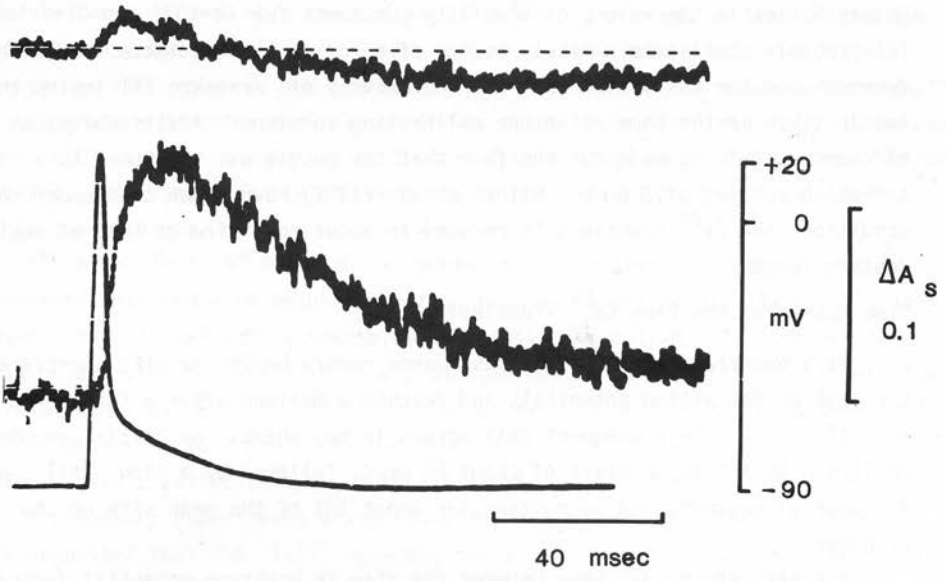

Fig.5: Absorbance records from a fibre injected with arsenazo III, showing the response to an action potential. Upper and middle traces show respectively, absorbance change at 532 nm, and at the wavelength pair 532-602 nm; lower trace, membrane potential. Absorbance traces were recorded through a low pass filter of time constant 0.5 msec. A suprathreshold depolarising pulse of 2 msec duration was used to initiate the action potential. Temp. 7.5°C. (From Miledi et al., 1977b).

becomes more acid during a twitch (Macdonald & Jöbsis, 1976), and this would give an absorbance change at 532-602 nm equivalent to a *decrease* in Ca^{2+} (Kendrick et al., 1977). Recordings of intracellular pH changes in fibres under identical conditions to those used for arsenazo III recording (Miledi, Parker & Schalow, unpublished data), using the indicator dye bromocresol purple (Macdonald & Jöbsis, 1977), show that the time course of the pH change is much slower than the arsenazo III response. There was a latency of about 12 msec (at 7°C) to the start of the pH transient, by which time the arsenazo III response had almost reached a peak. Any changes in arsenazo III light absorbance resulting from pH changes appear therefore to be small in comparison to Ca^{2+}-dependent changes, and in any case would affect only the falling phase of the Ca^{2+} transient.

Magnitude of the Free Ca^{2+} Transient

The peak size of the light response during a twitch was quite consistent between different experiments. The absorbance at 532-602 nm, measured in 49 fibres over a six-month period, was $\Delta A_S = 0.26$, with a standard deviation of

0.15 (Miledi et al., 1977b). This corresponds to a change in free Ca^{2+} concentration of 5.25 μM, using the calibration factor given earlier. Uncertainties in the values of stability constants for Ca-EGTA and Ca-arsenazo III probably constitute a likely source of error in this estimate. It also depends upon the assumption that the sensitivity of arsenazo III inside the muscle fibre is the same as in the calibrating solution. Additionally, an allowance should be made for the fact that the muscle was stretched to a striation spacing of 3.6 μm. Blinks et al. (1978) have shown that under these conditions the Ca^{2+} transient is reduced to about one-third of that at the resting length.

Time Course of the Free Ca^{2+} Transient

At a temperature of 7°C the absorbance record begins to rise shortly after the peak of the action potential, and reaches a maximum after a further 15-20 msec (Fig. 5). The subsequent fall occurs in two phases: an initial exponential decline with a time constant of about 50 msec, followed by a slow 'tail' lasting for several seconds, and accounting for about 10% of the peak size of the response.

A clear latency was seen between the rise in membrane potential during the action potential, and the onset of the light response (Fig. 6). Measuring from

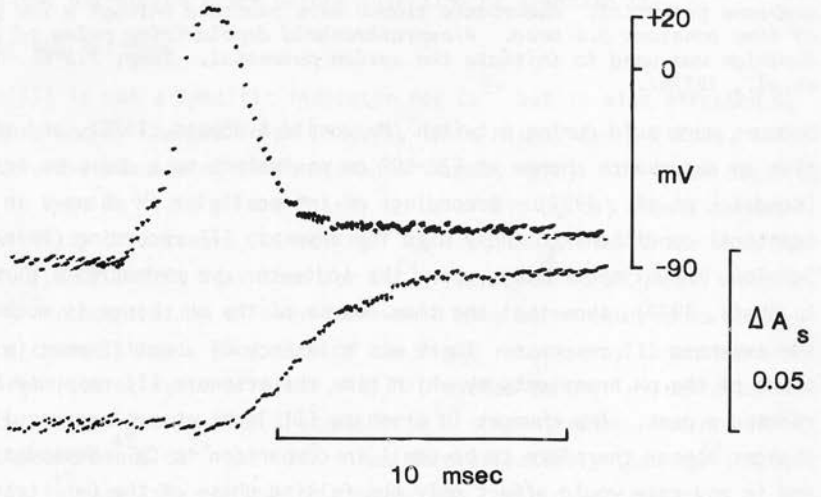

Fig. 6: Light response following an action potential. Upper trace, membrane potential; lower trace, change in absorbance at 532-602 nm. Optical recording time constant, 0.1 msec. Sixty sweeps were averaged, with stimuli given at 10 sec intervals. Temp. 5°C. (From Miledi et al., 1977b).

the 'foot' of the action potential, which corresponds closely with the threshold potential for Ca^{2+} release (Miledi et al., 1977b), a delay of 1.8 ±0.5 msec was found in twenty fibres at a temperature of 6-9°C. The response time of the dye is faster than 400 µsec (Brown, Cohen, DeWeer, Pinto, Ross & Salzberg, 1975; see Scarpa, this volume), and therefore cannot acount for the delay. Additionally, the temperature dependence of the delay was found to be high (mean Q_{10} 3.2 between 5 and 15°C), suggesting that it originates from a stage in excitation-contraction coupling, and is not readily attributable to the diffusion time of Ca^{2+} ions within the myoplasm.

The slow 'tail' of the light response is not apparent in records of Ca^{2+} transients obtained with aequorin in frog muscle (Blinks et al., 1978). It is unlikely that it reflects a true elevated level of myoplasmic Ca^{2+}, since this would be expected to give rise to a prolonged contracture. With slow repetitive stimulation (1 per sec) the 'tail' responses from successive twitches summate, and can give rise to a prolonged response which is as large as the peak attained during a single twitch (Miledi, Parker & Schalow, unpublished data).

Preliminary experiments using ruthenium red injected into muscle fibres have suggested that the 'tail' response may originate from those Ca^{2+} ions which are taken up by mitochondria. Ruthenium red is a specific inhibitor of mitochondrial Ca^{2+} binding and uptake (Vasington, Gazotti, Tiozzo & Carafoli, 1972; Alnaes & Rahamimoff, 1975), which appears to have little effect on Ca^{2+} release and uptake by the sarcoplasmic reticulum (Gillis, 1972). Injection of ruthenium red into fibres previously loaded with arsenazo III produced an almost complete abolition of the 'tail' component of the light response, whilst only slightly reducing the peak size of the transient during a single twitch (Miledi, Parker & Schalow, unpublished data).

Voltage Clamp Studies

Since it is necessary to block contraction by stretching the muscle for arsenazo III recording, there is no difficulty in leaving microelectrodes inserted in a fibre during stimulation. A simple two-point voltage clamp can then be used to control the membrane potential at the region used for absorbance measurements. For simplicity, the dye injection pipette was used as the current passing electrode, and pipettes with bevelled tips were able to pass adequate current for this purpose. To improve the performance of the voltage clamp, the inward sodium channels and delayed rectifying channels of the fibre were blocked by addition of tetrodotoxin (2 µg/ml) and tetraethyl ammonium chloride (30 mM) to the bathing solution.

Figure 7 illustrates absorbance records obtained by depolarising a fibre to

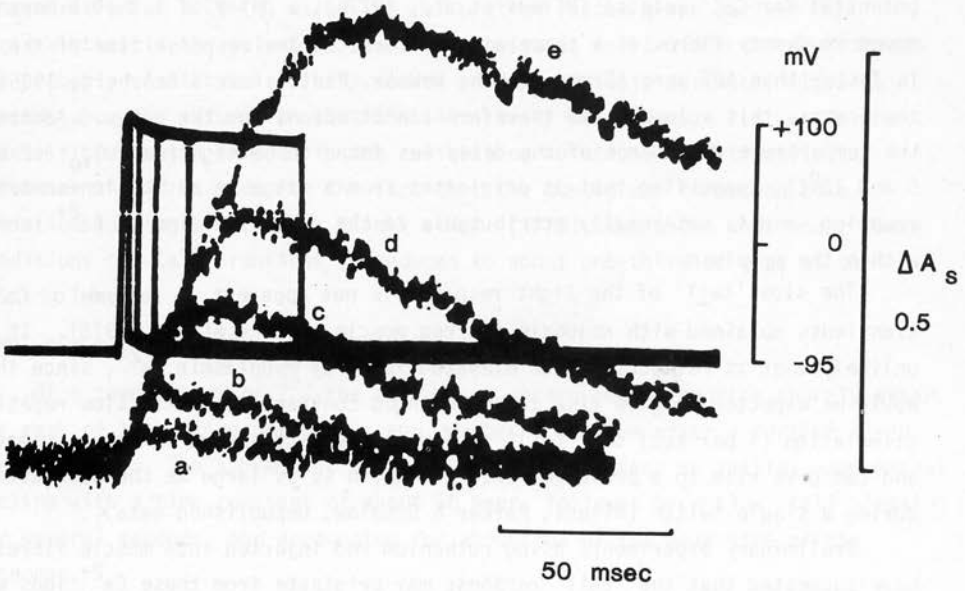

Fig.7: Light responses evoked by depolarising pulses of different durations. Upper trace, membrane potential; lower trace, change in absorbance at 532-602 nm. Superimposed records show pulses with durations of (a) 2, (b) 5, (c) 10, (d) 20 and (e) 50 msec. Temp. 6.5°C (from Miledi et al., 1977b).

+50 mV for varying durations. This membrane potential was greater than the value required to give a maximal Ca^{2+} release (Miledi et al., 1977b), and the size of the response was found to increase linearly with pulse duration up to about 20 msec. With longer duration pulses, the response began to 'round off', but this may have been at least partly due to saturation of the dye response, since the recorded absorbance change was close to the limiting value of ΔA_S (about 0.9). Following large free Ca^{2+} responses, the decline of the transient became slower and was linear, rather than exponential, suggesting that the uptake mechanism had become saturated.

Using short (10 msec) duration depolarising pulses of varying amplitudes, it was possible to determine the relation between membrane potential and Ca^{2+} release. The response size was found to be a graded function of potential between a threshold at -35 to -50 mV, and a maximum at about +10 mV (Miledi et al., 1977b).

Free Ca^{2+} Transients in Slow Muscle Fibres

Many muscles in the frog contain slow as well as twitch muscle fibres, which differ in their innervation, electrical and contractile properties and

fine structure (Lannergren, 1975). Slow fibres do not show action potentials but give graded slow contractions with nerve stimulation, and can maintain a prolonged contracture when depolarised. Records of Ca^{2+} transients have been obtained from slow fibres of the frog pyriformis muscle, using arsenazo III (Miledi et al., 1977c). These transients were very much slower than seen in twitch fibres, and their time course may play a major role in determining the time course of tension development and relaxation.

Measurements of absorbance changes of arsenazo III injected into slow fibres were made in the same way as for twitch fibres. Figure 8 illustrates records obtained by depolarising a fibre to different potentials. The size of the free Ca^{2+} transient was a graded function of membrane potential over a range of about -45 to 0 mV, and the time course of increase and decrease of the transient was slower than in twitch fibres (*cf.* Fig. 7).

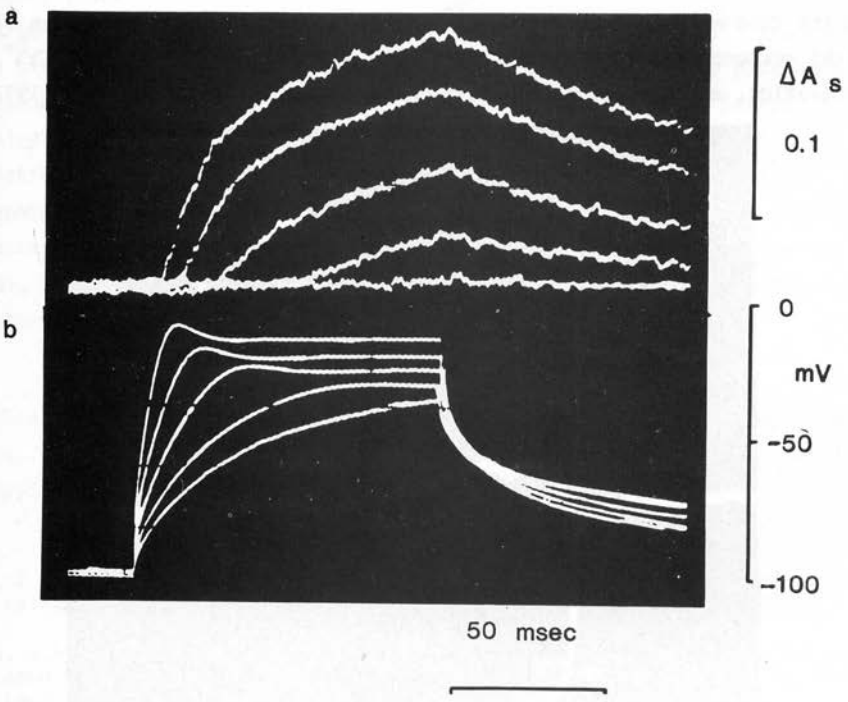

Fig. 8: Absorbance records from a slow muscle fibre of responses to depolarising pulses of different amplitudes. a, absorbance change at 532-602 nm. b, Membrane potential. Time constant for the optical traces was 10 msec. Five superimposed traces are shown. Stimuli were constant current pulses of 1 sec duration and varying amplitudes. Temp. 7°C. (From Miledi et al., 1977c).

Transmitter Activated Ca^{2+} Influx at the End-plate

There is evidence for an influx of Ca^{2+} ions across the post-junctional membrane of the neuro-muscular junction during transmitter action (Takeuchi, 1963; Katz & Miledi, 1969; Evans, 1974), and a postsynaptic Ca^{2+} influx has been recorded at the squid giant synapse using aequorin (Kusano, Miledi & Stinnakre, 1975). In arsenazo III-loaded muscle fibres, it is possible to record an increase in intracellular free Ca^{2+} at the end-plate region in response to either nerve stimulation, or to iontophoretic application of acetylcholine (Miledi et al., 1977a).

Figure 9 illustrates the absorbance change recorded in response to a single nerve impulse at a voltage-clamped end-plate. The time course of the light response is slower than the action potential response. This is probably because Ca^{2+} enters the fibre at a restricted area under the nerve terminal, thus resulting in a localised region of elevated free Ca^{2+} concentration, similar to the case with iontophoretic Ca^{2+} injection (*cf.* Fig. 4). The size of the light response was found to increase both with increasing external Ca^{2+} concentration, and with hyperpolarisation of the fibre (Miledi et al., 1977a).

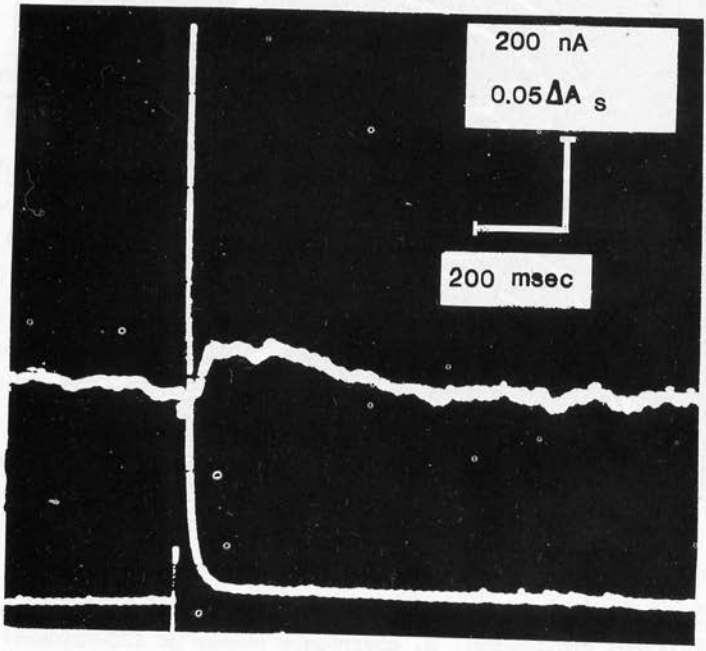

Fig. 9: Change in absorbance of arsenazo III at an end-plate following a single nerve stimulus. Upper trace, absorbance at 532-602 nm; lower trace, end-plate current. The fibre was voltage-clamped at -160 mV; bathing solution contained 12 mM Ca^{2+}. Time constant of the optical recording was 33 msec. Temp 8°C. A current pulse passed through a focal extracellular pipette was used to stimulate the nerve terminal.

CONCLUSIONS

Arsenazo III offers a complementary technique to aequorin for studying intracellular free Ca^{2+} transients in muscle, since each has advantages and disadvantages.

The main advantages of arsenazo III are that it is readily available, stable and can be easily injected into muscle fibres, and the relationship between free Ca^{2+} concentration and absorbance change is linear. The major disadvantages arise from the fact that the technique depends upon measuring very small changes in absorbance. Even with dual wavelength recording, it is difficult to ensure long-term stability of the absorbance baseline, and the system is best at detecting relatively rapid changes in free Ca^{2+} concentration. Muscle fibres present special problems because of movement artefacts during contraction, and it is necessary to reduce contraction by stretching the muscle, or by using hypertonic solutions.

Arsenazo III appears to be well suited for recording free Ca^{2+} transients during single twitches, since a good signal-to-noise ratio can be obtained, and the response time of the dye is rapid (relaxation time 2.4 msec; Scarpa, Tiffert & Brinley, 1977). For recording free Ca^{2+} transients during tetanic stimulation, or during long depolarisations, the dye suffers a number of disadvantages compared with aequorin: (i) it is difficult to prevent contraction artefacts, (ii) the 'tail' of the light response complicates interpretation of records, and (iii) the light absorbance changes occurring during tetani approach the saturation point of the dye.

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REFERENCES

ALNES, E. & RAHAMIMOFF, R. (1975). On the role of mitochondria in transmitter release from motor nerve terminals. J. Physiol., Lond. 248, 285-306.

BLINKS, J.R., RÜDEL, R. & TAYLOR, S.R. (1978). Calcium transients in isolated amphibian skeletal muscle fibres: detection with aequorin. J. Physiol., Lond. 277, 291-323.

BLIOCH, Z.L., GLAGOLEVA, I.M., LIBERMAN, E.A. & NENASHEV, V.A. (1968). A study of the mechanism of quantal transmitter release at a chemical synapse. J. Physiol., Lond. 199, 11-35.

BROWN, J.E., COHEN, L.B., DE WEER, P., PINTO, L.H., ROSS, W.N. & SALZBERG, B.M. (1975). Rapid changes of intracellular free calcium concentration. Detection by metallochromic indicator dyes in squid giant axon. Biophys. J. 15, 1155-1160.

CHANG, J.J. (1975). A new technique for beveling the tips of glass capillary micropipettes and microelectrodes. Comp. Biochem. Physiol. 52A, 567-570.

COHEN, S.M. & TYLER-BURT, C. (1977). ^{31}P nuclear magnetic relaxation studies of phosphocreatine in intact muscle: Determination of intracellular free magnesium. Proc. natn. Acad. Sci. U.S.A. 74, 4271-4275.

EBASHI, S. (1976). Excitation-contraction coupling. A. Rev. Physiol. 38, 293-313.

ENDO, M. (1977). Calcium release from the sarcoplasmic reticulum. Physiol. Rev. 57, 71-108.

EVANS, R.H. (1974). The entry of labelled calcium into the innervated region of the mouse diaphragm muscle. J. Physiol., Lond. 240, 517-533.

GILLIS, J.M. (1972). Le role du calcium dans le controle intracellularie de la contraction musculaire. University Catholique de Louvain: Thesis.

GORMAN, A.L.F. & THOMAS, M.V. (1978). Changes in a pace-maker neurone, measured with a metallochromic indicator dye arsenazo III. J. Physiol., Lond. 275, 357-376.

JOBSIS, F.F. & O'CONNOR, M.J. (1966). Calcium release and reapsorption in the sartorius muscle of the toad. Biochem. biophys. Res. Commun. 25, 246-252.

KATZ, B. & MILEDI, R. (1969). Spontaneous and evoked activity of motor nerve endings in calcium Ringer. J. Physiol., Lond. 203, 689-706.

KENDRICK, N.C. (1976). Purification of arsenazo III, a Ca^{2+} sensitive dye. Analyt. Biochem. 76, 487-501.

KENDRICK, N.C. RATZLAFF, R.W. & BLAUSTEIN, M.P. (1977). Arsenazo III as an indicator for ionised calcium in physiological salt solutions: its use for determination of the Ca ATP dissociation constant. Analyt. Biochem. 83, 433-450.

KUSANO, K., MILEDI, R. & STINNAKRE, J. (1975). Postsynaptic entry of calcium induced by transmitter action. Proc. R. Soc. B, 189, 49-56.

LANNERGREN, J. (1975). In: Basic Mechanisms of Ocular Motility and their Clinical Implications, ed. Lennerstrand, C. & Bach-y-Rita, P. Oxford: Pergamon Press.

MACDONALD, V.W. & JOBSIS, F.F. (1976). Spectrophotometric studies of the pH change during and after contractile activity. J. gen. Physiol. 68, 179-195.

MICHAYLOVA, V. & ILKOVA, P. (1971). Photometric determination of micro amounts of calcium with arsenazo III. Analyt. chim. Acta. 53, 194-198.

MILEDI, R., PARKER, I. & SCHALOW, G. (1977a). Calcium entry across the post-junctional membrane during transmitter action. J. Physiol., Lond. 268, 32-33P.

MILEDI, R., PARKER, I. & SCHALOW, G. (1977b). Measurement of calcium transients in frog muscle by the use of arsenazo III. Proc. R. Soc. B. 198, 201-210.

MILEDI, R., PARKER, I. & SCHALOW, G. (1977c). Calcium transients in frog slow muscle fibres. Nature, Lond., 268, 750-752.

SAVVIN, S.B. (1966). Arsenazo III. Atomizdat, Moscow.

SCARPA, A., TIFFERT, R. & BRINLEY, F.J. (1977). *In vivo* measurements of ionized Mg^{2+} and Ca^{2+} in single cells. In Biochemistry of Membrane Transport, ed. Semanza, G. & Carafoli, E., pp.552-566. Berlin: Springer-Verlag.

SUAREZ KURTZ, G. & PARKER, I. (1977). Birefringence signals and calcium transients in skeletal muscle. Nature, Lond., 270, 746-748.

TAKEUCHI, N. (1963). Effects of calcium on the conductance change of the end-plate membrane during the action of transmitter. J. Physiol., Lond. 167, 141-155.

VASINGTON, F.D., GAZZOTTI, P., TIOZZO, R. & CARAFOLI, E. (1972). The effect of ruthenium red on Ca^{2+} transport and respiration in rat liver mitochondria. Biochim. biophys. Acta 256, 43-54.