# Effects of membrane polarization on sarcoplasmic calcium release in skeletal muscle

By R. Miledi, F.R.S.,† S. Nakajima‡, I. Parker†
and T. Takahashi§

† *Department of Biophysics, University College London, Gower Street, London WC1E 6BT, U.K.*

‡ *Department of Biological Sciences, Purdue University, West Lafayette, Indiana 47907, U.S.A.*

Calcium release from the sarcoplasmic reticulum was investigated in voltage-clamped, tetrodotoxin-treated frog skeletal muscle fibres injected with arsenazo III. Short (5 ms) depolarizing pulses (test pulses) produced a transient change in arsenazo III absorption, signalling an increase in intracellular calcium ion concentration (calcium transient). Conditioning subthreshold depolarizations, which preceded the test pulse, potentiated the calcium transient triggered by the test pulse. Conditioning hyperpolarizations, applied either before or after the test pulse, inhibited the calcium transient. These effects of conditioning polarizations on the calcium transient may explain similar effects of subthreshold polarizations on muscle contraction that have previously been reported. The potentiating effect of subthreshold depolarizations was observed only when the test pulse was short (5 ms). The potentiating effect develops at $-48$ mV with a time constant of about 7 ms at 6.5 °C; this seems to be slower than that predicted by the potential spread from the surface along the tubular system. Thus, part of the effect could arise from the coupling process between tubular depolarization and calcium release.

## Introduction

The mechanism by which muscle membrane depolarization triggers the release of calcium from the sarcoplasmic reticulum is not well understood (Costantin 1975; Endo 1977). Some insights have been gained by experiments in which contraction was measured under various patterns of depolarizing pulses (Adrian *et al.* 1969; Bezanilla *et al.* 1971; Costantin 1974).

Recently, the dye arsenazo III has been shown to be a useful indicator for measuring the intracellular $Ca^{2+}$ concentration (Brown *et al.* 1975; DiPolo *et al.* 1976). We have used this technique to measure calcium concentration changes associated with contractile activation in twitch and slow muscle fibres (Miledi *et al.* 1977 *a, b*). The present paper is mainly concerned with the effects of subthreshold polarizations on the calcium released by a subsequent test pulse. By analysing the

§ Present address: Department of Physiology, Kyoto University, Faculty of Medicine, Kyoto, Japan.

data we obtained an estimate of the time course of the coupling process between membrane depolarization and the calcium release. Preliminary results were presented at a symposium (Miledi *et al.* 1981).

## Methods

### General procedures

The sartorius muscle of *Rana temporaria* was dissected out, and a sheet of thin-layered fibres was made under a dissecting microscope. The fibres were stretched to a sarcomere length of 3.0–3.6 µm, and the muscle was also stretched sideways by means of fine steel hooks. This produced regions where the sheet was only one to three fibres deep. Fibres in these thin regions were used for the experiments.

Arsenazo III (grade I from Sigma Chemical Co.) was iontophoretically injected through a bevelled microelectrode filled with 1 mg/ml arsenazo III plus 10 mM KCl (Miledi *et al.* 1977*a*), the injecting current generally being 100 nA. After being loaded with dye, the fibre was voltage-clamped at the injected area: the dye-injecting electrode served as the current electrode, and another microelectrode (KCl-filled), inserted at about 100 µm from the current electrode, was used as the potential electrode. Since the current electrode was filled with arsenazo III, and the potential was held at a hyperpolarized level by voltage clamp, the intracellular dye concentration increased gradually during the experiment. Thus, the background light intensity, at wavelength ($\lambda$) of 652 or 537 nm, which served as an indication of the arsenazo III concentration, was frequently monitored during the whole course of an experiment.

The composition of the bathing solution was NaCl 120 mM, KCl 2 mM, $CaCl_2$ 1.8 mM, HEPES–NaOH buffer 4 mM, with pH 7.2; and tetrodotoxin was added to a concentration of $(1–2) \times 10^{-6}$ g/ml. The bath temperature was 17.5–25 °C.

In the experiments designed to study the kinetics of potentiation at low temperature (figure 6), somewhat different procedures were employed. The preparations were sheets of fibres from *Xenopus* muscles (m. flexor brevis digiti V). Three intracellular microelectrodes (instead of two) were used. One was an arsenazo-injecting electrode (10 mM arsenazo III). After the dye injection, the electrode was withdrawn, and two electrodes, one for voltage recording (KCl-filled) and the other for current passing (potassium acetate-filled), were inserted in the dye-injected area for voltage clamping. The bath temperature in this series of experiments was 5.5–7 °C.

### Optical recording

The optical set-up was almost the same as that used for potential-probe dye experiments described previously (Nakajima & Gilai 1980). The main component was a Zeiss microscope. The dye-injected area of the fibre was illuminated with light from a quartz–halogen bulb, which was made quasi-monochromatic with an interference filter. The injected area was magnified by an objective lens ($\times$ 19, n.a. 0.5; or $\times$ 32, n.a. 0.40), and an adjustable slit was placed at the image plane so that only the light coming from an area about 80 µm length and about the whole

width of fibre was recorded. The approximate fibre width will be called diameter and the average value was 92 μm. The potential recording electrode was inserted either within or close to this optical recording area, whereas the current electrode was inserted outside this area. The light was collected by an eyepiece and measured by a photodiode (PV 100; EG&G Inc.). The signal was recorded from an oscilloscope, after automatic subtraction of d.c. offsets by means of a 'sample and hold' circuit.

In this paper arsenazo III signals are expressed in terms of either $\Delta A$ or $\Delta A / A_{652}$. The first, $\Delta A$, is absorbance change, namely $\Delta A \approx \Delta I / (2.3\,I)$, in which $\Delta I$ is change in light intensity emerging from the fibre caused by stimulation, and $I$ is the intensity emerging from the resting fibre. The term $A_{652}$ is the resting absorbance of the injected dye; namely $A_{652} = -\log_{10} (I_{652}/I_{0,652})$, in which $I_{652}$ is the intensity of the light emerging from the fibre after dye injection (at 652 nm) and $I_{0,652}$ is the light emerging from the fibre before dye injection. In most of fibres the value of $A_{652}$ was measured, and it was on average 0.09. The intracellular concentration of arsenazo III, calculated from eq. (4) of Nakajima & Gilai (1980) based on the known extinction coefficient of the dye (Kendrick *et al.* 1977), was on average 1.0 mM at an initial stage and 1.6 mM at a late stage of each experiment.

## RESULTS
### *Arsenazo III signals near threshold*

Figure 1a, b shows the time courses of arsenazo III absorption changes at a wavelength of 652 nm produced by long (200 ms) square-wave depolarizations. In this fibre the 'threshold' depolarization for the arsenazo signal is $-60$ mV (a). The average value of the threshold potential was $-54 \pm 2$ mV (mean $\pm$ s.e., $n = 7$).

Figure 1b shows the arsenazo signals elicited by larger depolarizations. There seem to be two phases in the time course of the signal, one a rapidly increasing phase reaching a peak at 25 ms, and the other a plateau phase, either staying unchanged or creeping up slowly. Sometimes a small dip separated these two phases (b), but often the transition between them was smooth. In agreement with previous work (Miledi *et al.* 1977a; Baylor *et al.* 1979b), the relation between the potential and signal size was very steep for potentials close to threshold; an e-fold signal change (measured at 100–220 ms) occurred for potential change of $3.3 \pm 0.3$ mV (mean $\pm$ s.e., 7 fibres). The rate of rise of the arsenazo signal $d(\Delta A / A_{652})/dt$ was also a steep function of the potential, and an e-fold change occurred with potential change of $3.6 \pm 0.3$ mV (mean $\pm$ s.e., 7 fibres).

When the wavelength was changed from 652 to 537 nm, the polarity of the arsenazo signal was reversed (figure 1c). However, the signal at 537 nm is not a perfect mirror image of that at 652 nm (the magnitude of depolarization in (c) was almost the same as that of the larger depolarization in (b)). At 537 nm, the signal does not have the slow creep, which is obviously present at the larger depolarization at 652 nm.

The wavelength dependency (spectrum) of the arsenazo signal was determined by means of a limited number of interference filters. In figure 2c, square symbols

represent the spectrum measured with short (3.6 ms) depolarizing pulses (sample records in $(a)$), whereas circles are measured with long (220 ms) depolarizing pulses (sample records in $(b)$). The general shape of the spectrum measured with the short pulses is similar to the difference spectrum when the intracellular $Ca^{2+}$ was expected to increase in photoreceptors or in molluscan neurons (Brown *et al.* 1977; Ahmed & Connor 1979). Thus, there are two peaks (squares of figure 2$c$), a larger one at 650 and the smaller one at 600 nm, with isosbestic point near 560 nm. Thus, it seems that arsenazo III signals produced by the short depolarizations represent an increase in the myoplasmic $Ca^{2+}$ concentration (the calcium transient).
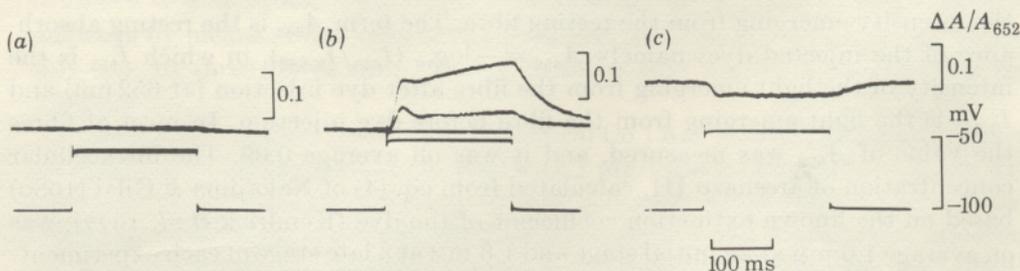


FIGURE 1. Absorption changes (upper traces) of an arsenazo III injected muscle fibre treated with TTX. Upward direction signals an increase in absorption. Depolarizing pulses (lower traces) were applied under voltage clamp. Calibration bars for absorption refer to $\Delta A/A_{652}$ (i.e. changes in absorbance at a given wavelength divided by the absorbance of the injected dye at $\lambda = 652$ nm). In $(a)$ and $(b)$, $\lambda = 652$ nm. In $(c)$, $\lambda = 537$ nm. Fibre diameter 91 μm. Temperature 21 °C.

On the other hand, the spectrum with longer pulses (circles in figure 2$c$) is somewhat different from the one with short pulses. The 600 nm peak is not obvious and the isosbestic point is shifted to the left. This fact, as well as the observation that the time course of the signals at 652 nm is not a perfect mirror image of that at 537 nm (figure 1), indicates that the signals produced by long depolarizations are contaminated by some phenomena other than calcium changes (including perhaps small movement artefacts).

Baylor *et al.* (1979$a$, $b$) reported the existence of two kinds of arsenazo III signals (one reflecting calcium, the other something else) if measured with the light polarized at 0° to the fibre long axis, whereas, with light polarized at 90° to this axis the predominant signals were the calcium transient. For two fibres we studied effects of polarized light on arsenazo III signals. At a wavelength of 652 nm, we could not see any measurable dichroism. This is probably because, as noted in Baylor *et al.* (1979$a$), the dichroic effect is not expected to be prominent at the high concentrations of arsenazo used in our experiments.

*Effects of conditioning membrane polarization*

This section is concerned with effects of conditioning subthreshold membrane polarizations on the calcium transient. Figure 3$a$ illustrates the pattern of the conditioning and test pulse sequence. The holding potential is $V_1$, and the conditioning depolarization (or hyperpolarization) is $V_2$ and lasts for a time $t_1$. The
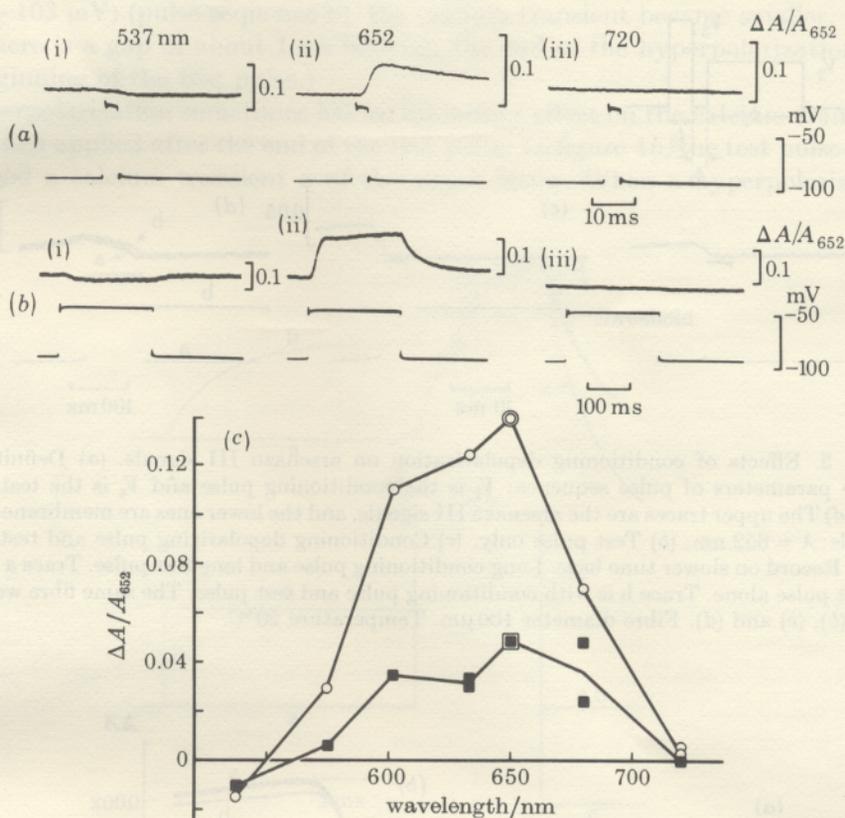
FIGURE 2. Wavelength dependency of arsenazo III signals. (*a*) Arsenazo signals produced by short depolarizing pulses. (*b*) Arsenazo signals produced by long pulses. Upper trace in each frame is arsenazo III signal (vertical calibration bar $\Delta A/A_{652}$) at wavelength indicated at top of each column, and lower trace is membrane potential. (*c*) The arsenazo III signal is plotted against wavelength: filled squares, short depolarizing pulses (sample records in (*a*)); open circles: long depolarizing pulses (sample records in (*b*)). The amplitudes of arsenazo signals changed gradually during the experiments, probably because of changes of intracellular dye concentration. This effect was corrected as follows: measurements at several wavelengths were bracketed by measurement at 652 nm, and the values were normalized in reference to the value at 652 nm, taken at the initial stage of experiments. No correction for this effect was made in the sample records. Fibre diameter 73 µm. Temperature 20 °C.

potential of the test pulse is $V_3$ and its duration is $t_3$. Between the conditioning pulse and the test pulse there is a gap $t_2$.

In figure 3*b*, *c*, we show effects of conditioning depolarizations. The calcium transient obtained by a test pulse alone (figure 3*b*) ($V_3 = -26$ mV, $t_3 = 5$ ms) was small (0.0095 in the unit of $\Delta A/A_{652}$). In figure 3*c*, a conditioning depolarization ($V_2 = -58$ mV) of 29 ms duration, which by itself was below the threshold for calcium release, preceded the test pulse. This pulse sequence now produced a larger calcium transient (0.026) than with the test pulse alone. (A small gap, $t_3$, of 0.8 ms
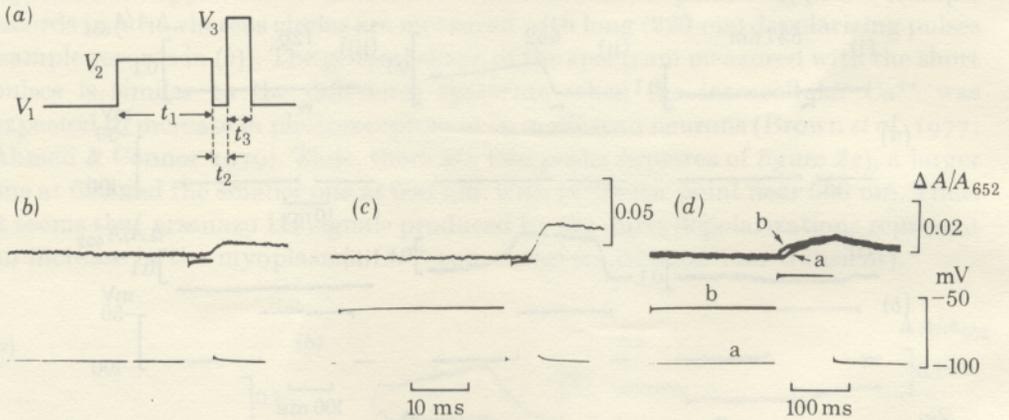
FIGURE 3. Effects of conditioning depolarization on arsenazo III signals. (a) Definition of the parameters of pulse sequence; $V_2$ is the conditioning pulse and $V_3$ is the test pulse. (b–d) The upper traces are the arsenazo III signals, and the lower ones are membrane potentials; $\lambda = 652$ nm. (b) Test pulse only. (c) Conditioning depolarizing pulse and test pulse. (d) Record on slower time base. Long conditioning pulse and long test pulse. Trace a is with test pulse alone. Trace b is with conditioning pulse and test pulse. The same fibre was used in (b), (c) and (d). Fibre diameter 100 µm. Temperature 20 °C.
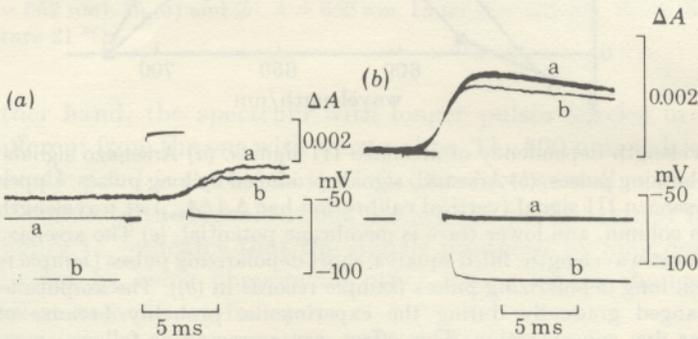


FIGURE 4. Effects of hyperpolarization on arsenazo III signals. Upper traces are arsenazo III signals, and lower traces are membrane potentials; $\lambda = 652$ nm. (a) A hyperpolarizing conditioning pulse preceded the test pulse. Trace a is with test pulse alone, and trace b is with hyperpolarization pulse followed by the test pulse. (b) Test pulse was followed by a hyperpolarization: trace a is with test pulse alone, and trace b is with the test pulse followed by the hyperpolarization. There is a small gap between the end of test pulse and the beginning of hyperpolarization. Fibre diameter 100 µm. Temperature 25 °C.

was interposed between the end of the conditioning pulse and the beginning of the test pulse. This gap was necessary, since otherwise it is difficult to produce the same shape of the test pulse in the sequence as of the test pulse alone.)

The effect of a conditioning hyperpolarization is illustrated in figure 4a. Two records are superimposed. The pulse sequence a consists of a test depolarization alone ($V_1 = -63$ mV; $V_3 = -4$ mV; $t_3 = 2.9$ ms), which resulted in a calcium

transient a. When the test pulse was preceded by a hyperpolarization ($V_2 = -103$ mV) (pulse sequence b), the calcium transient became smaller. (Note that there is a gap of about 1 ms between the end of the hyperpolarization and the beginning of the test pulse.)

Hyperpolarization sometimes has an inhibitory effect on the calcium transient, even when applied after the end of the test pulse. In figure 4b, the test pulse alone produced a calcium transient a of the upper beam. When a hyperpolarization
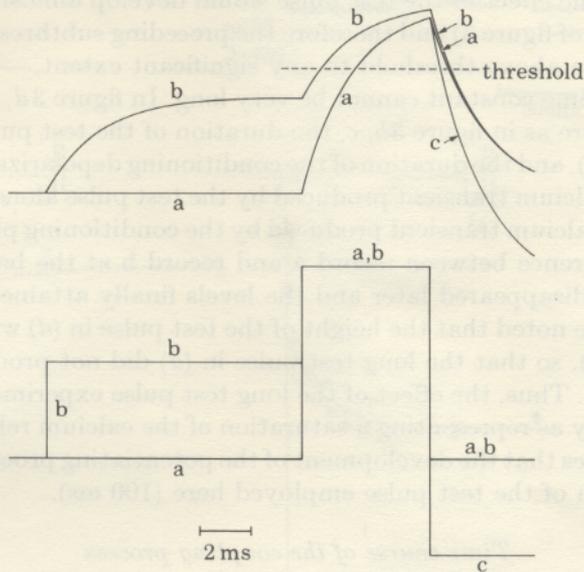


FIGURE 5. A linear model of development and recovery of potentiation. Lower traces are membrane potentials, and upper traces are time courses of potentiation. See text.

($V_3 = -113$ mV) was applied after the end of the test pulse, the calcium transient became smaller (trace b) (the gap between the end of the test pulse and the beginning of the hyperpolarization was 0.4 ms).

The subthreshold effect of conditioning polarizations that we observed can be explained by reference to the hypothetical linear model illustrated in figure 5. The lower traces are the pulse sequence applied, and the upper traces represent the time course of the potentiating process. In the sequence of traces a, only the test pulse is applied, and the potentiating process is assumed to develop exponentially, and when the potentiating process reaches the threshold (horizontal dotted line), the calcium release starts. When a conditioning depolarization is applied, a subthreshold potentiating process develops (b), and the subsequent test pulse produces a potentiating process, which starts from an already elevated level. Thus, even with the same time constant, it will reach the threshold level earlier and stay above the threshold longer (b). Thus, the area above the threshold will become larger than for the test pulse alone. (To put it another way, when the conditioning depolarization is applied, the potentiating process left over from the conditioning depolarization summates with the potentiating process initiated by the test pulse.)

The effect of conditioning hyperpolarizations, even after the end of the test pulse, is similarly explained. In this case, the after-hyperpolarization causes the potentiating process to subside more quickly (see curve c), and this effect will make the area above the threshold smaller.

In the model of figure 5, the time constant of the potentiating process was arbitrarily set to be 2 ms. The experiments so far described cannot tell whether this is correct. Nevertheless, we can make a rough estimate of the time constant.

First, the time constant cannot be very short. If it were very short, say of the order of 0.1 ms, the effect of the test pulse would develop almost instantaneously (on the time scale of figure 5) and therefore the preceding subthreshold pulse would not affect the area above threshold to any significant extent.

Secondly, the time constant cannot be very long. In figure $3d$, which was taken from the same fibre as in figure $3b$, $c$, the duration of the test pulse ($t_3$) was made very long (100 ms), and the duration of the conditioning depolarization was 220 ms. Record a is the calcium transient produced by the test pulse alone, whereas record b represents the calcium transient produced by the conditioning plus the test pulse. There was a difference between record a and record b at the beginning, but the initial difference disappeared later and the levels finally attained are almost the same. It should be noted that the height of the test pulse in ($d$) was set lower than that in ($b$) and ($c$), so that the long test pulse in ($d$) did not produce a very large calcium transient. Thus, the effect of the long test pulse experiment ($d$) cannot be interpreted simply as representing a saturation of the calcium release mechanism. The result indicates that the development of the potentiating process is much faster than the duration of the test pulse employed here (100 ms).

### Time course of the coupling process

All the experiments described so far were done on frog sartorius muscles at a temperature of 17.5–25 °C. This section deals with an attempt to measure the time course of the coupling process for calcium release, the experiments being done at low bath temperature (5.5–7 °C) on *Xenopus* muscle (see methods section).

In figure $6c$ the calcium transient was plotted against the duration of the conditioning depolarization ($t_1$), other parameters being kept constant. Sample records of the experiments are displayed in ($a$ i) and ($a$ ii). There is always a gap of 0.2 ms (not visible in the photographs) between the end of the conditioning pulse and the beginning of the test pulse. Figure $6c$ shows that the potentiating effect of the conditioning depolarization develops with a time constant of 5.6 ms. (There seem to be two components in the time course: a fast small component and a slow main component. In determining the time constant, we disregarded the point at $t_1 = 0$ (square symbol). Thus, the value obtained would correspond to that of the slower component.) If it is assured that the relation between the level of the potentiating process achieved by the conditioning pulse and the calcium transient size elicited by the test pulse is fairly linear, the time constant of the potentiating effect (5.6 ms) could correspond to the time constant of the onset of the coupling process brought about by the conditioning depolarization. In three fibres (7 runs altogether), the mean value of the time constant at 6.5 °C was $6.7 \pm 2.1$ ms (mean $\pm$ s.d.) with conditioning depolarizations of $-48 \pm 14$ mV (mean $\pm$ s.d.). (It

may be noted that the conditioning depolarization, $V_2$, of $-48$ mV was still subthreshold for calcium release at low temperature (cf. Costantin 1974). Other conditions were $V_1 = -90$ to $-116$ mV, $V_3 = -19$ to $+17$ mV, and $t_3 = 5$–7 ms.)

In these experiments, it was sometimes observed that the magnitude of the test pulse varied slightly (by less than 2 mV) depending on the duration of the
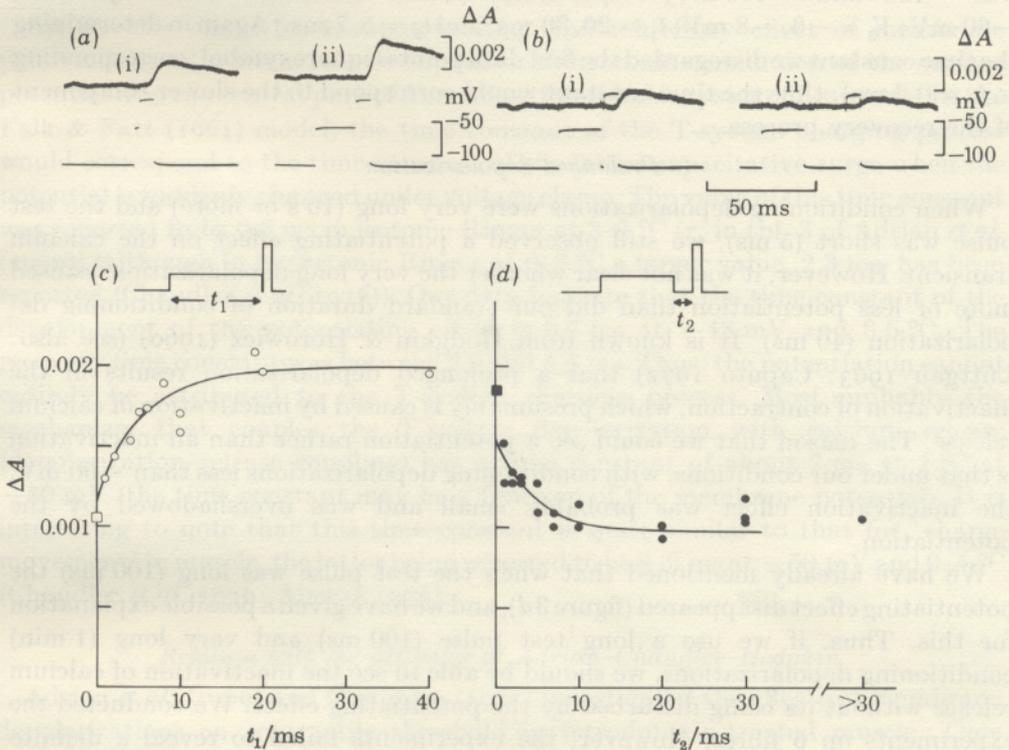


FIGURE 6. Time course of potentiation. (c) Development of potentiation. The arsenazo III signal was plotted against the duration of conditioning depolarization ($t_1$). There were always short gaps (0.2 ms) between the end of the conditioning pulse and the beginning of the test pulse. The continuous curve is: $\Delta A = 0.00082\{1 - \exp(-t/5.6)\} + 0.00117$. (d) Recovery from potentiation. The arsenazo III signal was plotted against the length of the gap ($t_2$) between the conditioning pulse and the test pulse. The continuous curve is: $\Delta A = 0.00097 + 0.00059$ $\exp(-t/4.5)$. The symbol $> 30$ means that no conditioning depolarization was applied. Slow spontaneous changes of the arsenazo III signal during the course of an experiment were corrected for by bracketing several measurements with the standard measurement (open squares in (c) and the point at $> 30$ in (d)). (a) Sample records of the experiment in (c). (b) Sample records of the experiment in (d). Fibre diameter 68 μm. Temperature 7 °C.

conditioning depolarization. This phenomenon is probably due to resistance changes (with hysteresis) in the current electrode caused by the conditioning current, resulting in slightly imperfect clamping. We should have used current electrodes of lower resistance, to make the clamping more perfect. Nevertheless, this source of error did not change the time constant of potentiation significantly.

In figure 6d, the relation between the calcium transients and the time interval

between the conditioning pulse and the test pulse ($t_2$) was plotted, keeping other parameters constant. This curve, reflecting subsidence of the potentiation, would represent the time course of the recovery from the subthreshold coupling process developed by the conditioning depolarization. The time constants of the recovery in two fibres were 2.0 and 4.5 ms when the membrane potential during recovery was $-100$ and $-116$ mV, respectively. (Other conditions were: $V_2 = -48$, $-60$ mV; $V_3 = -6, -8$ mV; $t_1 = 20, 30$ ms; and $t_3 = 5, 7$ ms.) Again in determining the time constant we disregarded the first data points (square symbol; corresponding to $t_2 = 0.2$ ms); thus the time constant would correspond to the slower component of the recovery process.

### Prolonged depolarization

When conditioning depolarizations were very long (10 s or more) and the test pulse was short (5 ms), we still observed a potentiating effect on the calcium transient. However, it was not clear whether the very long depolarizations caused more or less potentiation than did our standard duration of conditioning depolarization (40 ms). It is known from Hodgkin & Horowicz (1960) (see also: Lüttgau 1963; Caputo 1972) that a prolonged depolarization results in the inactivation of contraction, which presumably is caused by inactivation of calcium release. The reason that we could see a potentiation rather than an inactivation is that under our conditions, with conditioning depolarizations less than $-60$ mV, the inactivation effect was probably small and was overshadowed by the potentiation.

We have already mentioned that when the test pulse was long (100 ms) the potentiating effect disappeared (figure 3$d$), and we have given a possible explanation for this. Thus, if we use a long test pulse (100 ms) and very long (1 min) conditioning depolarizations, we should be able to see the inactivation of calcium release without its being disturbed by the potentiating effect. We conducted the experiments on 6 fibres. However, the experiments failed to reveal a definite inactivation curve because of large scatter in the results.

## DISCUSSION

### Relation with contractile inactivation

There have been reports that conditioning depolarizations (duration 100 ms (Bezanilla et al. 1971) or 10 s (Bastian & Nakajima 1974)) produce an enhancement of twitch tension initiated by subsequent short pulses (see Caputo & DiPolo (1978), for barnacle muscle fibres). These results are in contrast with the result of the potassium depolarization experiments by Hodgkin & Horowicz (1960), who never saw the potentiation; instead, they observed only a decrease of contractile response when conditioning depolarizations (by high K solution) were applied. This apparent contradiction has been resolved by the present experiments on calcium release. Our results show that the time course of the potentiation of calcium release is on the order of 5 ms. Thus, the summation becomes evident only when the test pulse is relatively short (ca. 5 ms). When the test depolarizations are long (more than 100 ms, such as those used by Hodgkin & Horowicz (1960)), the initial

potentiating effect of calcium release is overshadowed by the subsequent larger calcium release, which reaches the same level regardless of the initial condition (figure 3d). Furthermore, when potassium depolarization is used, the speed of depolarization is too slow to reveal the initial phase of facilitation.

### Relation with T-system conduction

An obvious cause for the potentiating and inhibitory effect of membrane polarizations on calcium release is passive electrotonic conduction of the surface potential into the central part of fibre along the tubular system (T-system). In the Falk & Fatt (1964) model, the time constant of the T-system charging process would correspond to the time constant of the initial capacitative surge when the potential is suddenly changed under voltage clamp. The value of this time constant was reported to be 0.6 ms in isotonic Ringer at 3–6 °C ($\tau_s$ in tbl. 3 of Adrian *et al.* (1970)) (although in hypertonic Ringer at 0–2 °C a larger value, 2.3 ms, has been reported (Chandler *et al.* 1976)). Our data indicate that the time constant of the development of the potentiating effect is 6.7 ms at −48 mV and 6.5 °C. The recovery time constant was between 2.0 and 4.5 ms. Thus, the potentiation cannot entirely be attributed to the T-system charging process. Most probably the mechanism that couples the T-system depolarization with calcium release (depolarization–release coupling) has a time constant of about 7 ms at 7 °C at −50 mV (the time constant may be a function of the membrane potential). It is interesting to note that this time constant is quite similar to that for 'charge movement' in muscle, the latter being reported to be 8–9 ms at −50 mV and 0–5 °C (Chandler *et al.* 1976; Almers 1976).

### Relation with the activator of Adrian–Chandler–Hodgkin

Adrian *et al.* (1969) and Costantin (1974) investigated the effects of membrane depolarizations on contractile threshold in tetrodotoxin-treated muscle. They determined the strength–duration relation for the threshold. They also observed that conditioning hyperpolarizations or depolarizations, applied either before or after the test pulse, had inhibitory or potentiating effects on the contractile activation. Adrian *et al.* (1969) interpreted their results in terms of a simple kinetic model, in which depolarization produces an increase in the concentration of a hypothetical substance *activator*, and contraction is initiated when the *activator* concentration reaches a certain level.

At present, it is not established what constitutes the *activator* mentioned in Adrian *et al.* (1969). It could be that the *activator* is the calcium itself released from the sarcoplasmic reticulum. Potentiation would occur because the conditioning depolarization releases some amount of calcium, and the subsequent release of calcium by the test pulse summates with the previous one to reach the threshold (for contraction). Alternatively, the *activator* could be the coupling mechanism that leads to the calcium release (namely, some process prior to the calcium release). This coupling process could occur even with depolarizations subthreshold for calcium release, so that the subsequent process initiated by the test pulse summates with the previous one to reach the threshold (for calcium release). So long as investigations are confined to recording mechanical events, it is difficult

to decide which of the above alternatives is correct. Thus, Adrian *et al.* (1969) were non-committal. Costantin (1974) tried to interpret some of his results in terms of changes in the calcium concentrations (p. 672 of his paper), whereas Adrian (1978), from analysing the charge movement, suggested that the activator could reflect the coupling process.

Our present results (figures 3, 4) have clearly shown that a conditioning hyper- or depolarization, which by itself has no detectable effect on the arsenazo signal, has an inhibitory or potentiating effect on the subsequent release of calcium. Therefore, there is a subthreshold summation of the coupling mechanism for the calcium release. This effect would play a substantial role in determining the overall kinetics of *activator* of Adrian *et al.* (1969). However, since the time course of the arsenazo signals is also comparable to the time course of our potentiating effects, it is likely that the *activator* of Adrian *et al.* (1969) is composed of at least two components (in addition to the tubular conduction), one the coupling mechanism for calcium release, and the other calcium concentration changes in myoplasm. In other words, the speed of neither of the two is rapid enough to be negligible for determining the overall time course.

In summary, the present experiments allow us to divide the excitation–contraction coupling system into three parts, when action potentials are blocked: (1) T-system transmission (time constant less than 1 ms in isotonic Ringer); (2) coupling between T-system depolarization and calcium release (time constant of about 7 ms at 7 °C); and (3) building up of the intracellular calcium concentration resulting in the initiation of movement.

## References

Adrian, R. H. 1978 Charge movement in the membrane of striated muscle. *A. Rev. Biophys. Bioengng* **7**, 85–112.

Adrian, R. H., Chandler, W. K. & Hodgkin, A. L. 1969 The kinetics of mechanical activation in frog muscle. *J. Physiol., Lond.* **204**, 207–230.

Adrian, R. H., Chandler, W. K. & Hodgkin, A. L. 1970 Voltage clamp experiments in striated muscle fibres. *J. Physiol., Lond.* **208**, 607–644.

Ahmed, Z. & Connor, J. A. 1979 Measurement of calcium influx under voltage clamp in molluscan neurones using the metallochromic dye arsenazo III. *J. Physiol., Lond.* **286**, 61–82.

Almers, W. 1976 Differential effects of tetracaine on delayed potassium channels and displacement currents in frog skeletal muscle. *J. Physiol., Lond.* **262**, 613–637.

Bastian, J. & Nakajima, S. 1974 Action potential in the transverse tubules and its role in the activation of skeletal muscle. *J. gen. Physiol.* **63**, 257–278.

Baylor, S. M., Chandler, W. K. & Marshall, M. W. 1979a Arsenazo III signals in frog muscle. *Biophys. J.* **25**, 141a.

Baylor, S. M., Chandler, W. K. & Marshall, M. W. 1979b Arsenazo III signals in singly dissected frog twitch fibres. *J. Physiol., Lond.* **287**, 23P–24P.

Bezanilla, F., Caputo, C. & Horowicz, P. 1971 Voltage clamp activation of contraction in short striated muscle fibers of the frog. *Acta cient. venez.* **22** (2), 72–74.

Brown, J. E., Brown, P. K. & Pinto, L. H. 1977 Detection of light-induced changes of intracellular ionized calcium concentration in *Limulus* ventral photoreceptors using arsenazo III. *J. Physiol., Lond.* **267**, 299–320.

Brown, J. E., Cohen, L. B., DeWeer, P., Pinto, L. H., Ross, W. N. & Salzberg, B. M. 1975 Rapid changes of intracellular free calcium concentration detected by meallochromic indicator dyes in squid giant axon. *Biophys. J.* **15**, 1155–1160.

Caputo, C. 1972 The time course of potassium contractures of single muscle fibres. *J. Physiol., Lond.* **223**, 483–505.

Caputo, C. & DiPolo, R. 1978 Contractile activation phenomena in voltage-clamped barnacle muscle fiber. *J. gen. Physiol.* **71**, 467–488.

Chandler, W. K., Rakowski, R. F. & Schneider, M. F. 1976 A non-linear voltage dependent charge movement in frog skeletal muscle. *J. Physiol., Lond.* **254**, 245–283.

Costantin, L. L. 1974 Contractile activation in frog skeletal muscle. *J. gen. Physiol.* **63**, 657–674.

Costantin, L. L. 1975 Contractile activation in skeletal muscle. *Prog. Biophys. molec. Biol.* **29**, 197–224.

DiPolo, R., Requena, J., Brinley Jr, F. J., Mullins, L. J., Scarpa, A. & Tiffert, T. 1976 Ionized calcium concentrations in squid axons. *J. gen. Physiol.* **67**, 433–467.

Endo, M. 1977 Calcium release from the sarcoplasmic reticulum. *Physiol. Rev.* **57**, 71–108.

Falk, G. & Fatt, P. 1964 Linear electrical properties of striated muscle fibres observed with intracellular electrodes. *Proc. R. Soc. Lond.* B **160**, 69–123.

Hodgkin, A. L. & Horowicz, P. 1960 Potassium contractures in single muscle fibres. *J. Physiol., Lond.* **153**, 386–403.

Kendrick, N. C., Ratzlaff, R. W. & Blaustein, M. P. 1977 Arsenazo III as an indicator for ionized calcium in physiological salt solutions: its use for determination of the CaATP dissociation constant. *Analyt. Biochem.* **83**, 433–450.

Lüttgau, H. C. 1963 The action of calcium ions on potassium contractures of single muscle fibres. *J. Physiol., Lond.* **168**, 679–697.

Miledi, R., Nakajima, S. & Parker, I. 1981 Effects of conditioning membrane potentials on calcium transients in skeletal muscle fibers. In *The regulation of muscle contraction, excitation–contraction coupling* (ed. A. D. Grinnell & M. A. B. Brazier), pp. 175–180. New York: Academic Press.

Miledi, R., Parker, I. & Schalow, G. 1977a Measurement of calcium transients in frog muscle by the use of arsenazo III. *Proc. R. Soc. Lond.* B **198**, 201–210.

Miledi, R., Parker, I. & Schalow, G. 1977b Calcium transients in frog slow muscle fibres. *Nature, Lond.* **268**, 750–752.

Nakajima, S. & Gilai, A. 1980 Action potentials of isolated single muscle fibers recorded by potential-sensitive dyes. *J. gen. Physiol.* **76**, 729–750.