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CALCIUM TRANSIENTS STUDIED UNDER VOLTAGE-CLAMP CONTROL IN FROG TWITCH MUSCLE FIBRES

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SUMMARY

1. Intracellular calcium transients were recorded from frog twitch muscle fibres in response to voltage-clamped depolarizing pulses, using arsenazo III as an intracellular calcium monitor. The object was to investigate the time- and voltage-dependent characteristics of the coupling process between membrane depolarization and calcium release from the sarcoplasmic reticulum (s.r.)

2. To examine the extent to which the T-tubule membrane potential was controlled during clamp pulses, the dye NK 2367 was used as an optical probe of tubular potential. This indicated that the tubular time constant is about 0·6 msec.

3. Strength–duration curves were obtained for depolarizing pulses required to give both threshold mechanical contraction and calcium signal. Curves measured in these two ways were closely similar.

4. Changes in holding potential altered the strength–duration curve for calcium release so that at more positive holding potentials a shorter pulse was needed to obtain a response for any given pulse amplitude.

5. A latency of a few milliseconds was observed between the onset of depolarization and the initial rise of the calcium signal. This became shorter with stronger depolarizations, but approached a minimum at potentials above about +25 mV.

6. Subthreshold depolarizations applied before a test pulse increased the size and decreased the latency of the calcium signal. Conditioning hyperpolarizations had opposite effects.

7. The rate of build-up of potentiation or depression of response size seen with subthreshold de- and hyperpolarizing conditioning pulses was examined using conditioning pulses of different durations. For both pulses this process showed a time constant of about 3 msec (at 10 °C).

8. The rate of decay of potentiation or depression was similarly measured, using a gap of variable duration between conditioning and test pulses. For both de- and hyperpolarizing pulses this showed a time constant of about 5 msec (10 °C).

9. The relationship between conditioning pulse potential, and the size of calcium signal elicited by a following test pulse was non-linear.

10. Subthreshold pulses immediately following a brief test pulse affected the size of the calcium signal in a similar way to preceding conditioning pulses.

11. The relationship between potential and size of the calcium signal was examined

using pulses of 3 and 20 msec duration. With the long pulse the relation was roughly sigmoid, but with the short pulse continued to rise even at strongly positive potentials.

12. The results are discussed in terms of a model in which the exponential build-up of a hypothetical coupler in the excitation–contraction (e.–c.) coupling process is presumed to lead to calcium release when a threshold level is exceeded.

INTRODUCTION

A critical link in the coupling of excitation to contraction in twitch muscle is the process whereby depolarization of the T-tubules leads to release of calcium from the sarcoplasmic reticulum (s.r.) (for reviews see; Endo, 1977; Costantin, 1975; Caputo, 1978; Stephenson, 1981; Grinnell & Brazier, 1981). At present this process is poorly understood, probably because of a lack of techniques capable of directly attacking the problem. However, recent developments in two fields show considerable promise. One is the use of intracellular indicators to monitor the calcium released from the s.r. (Miledi, Parker & Schalow, 1977*a*, *b*, 1979; Miledi, Nakajima & Parker, 1981*a*; Miledi, Nakajima, Parker & Takahashi 1981*b*; Miledi, Parker & Zhu, 1982, 1983; Blinks, Rudel & Taylor, 1978; Kovacs, Rios & Schneider, 1979; Palade & Vergara, 1982). The second is the recording of non-linear charge movements, which are thought to reflect an initial step in the coupling process (Schneider & Chandler, 1973; Schneider, 1981; Adrian, 1978).

Our approach has been to make a quantitative examination of the 'input–output' relationships of the coupling process. Here, the input is the membrane potential, which was controlled by a voltage clamp. The output is the release of calcium from the s.r., and this was monitored using intracellular arsenazo III. By studying the time and voltage-dependent characteristics of the calcium release, considerable information could be obtained about the intervening stages between depolarization and calcium release.

We have previously proposed a simple linear model to explain the potentiation and depression of the calcium transient observed when test depolarizations are preceded by subthreshold conditioning pulses (Miledi *et al.* 1981*b*). In the present paper this idea is developed further, and with slight elaborations it is able to explain the observed properties of calcium release for both sub- and suprathreshold pulses.

The basis of the model is to suppose that depolarization of a muscle fibre leads to the build up of a hypothetical coupler in the excitation–contraction (e.–c.) coupling process between depolarization and calcium release from the s.r. The steady-state level of coupler is proportional to the membrane potential but, following step changes in potential, the build up and decay of coupler proceed with exponential time courses. The level of coupler must exceed a threshold value before release of calcium begins and for levels above threshold the rate of release of calcium is presumed to be a linear function of coupler level. This model is illustrated in Fig. 1 for various stimulus conditions.

Adrian, Chandler & Hodgkin (1969*a*) proposed a rather similar model, in which the build up of a hypothetical 'activator' was introduced to explain the strength–duration curve for contractile activation. However, in their case, only mechanical contraction was observed and it was not clear whether the activator represented the

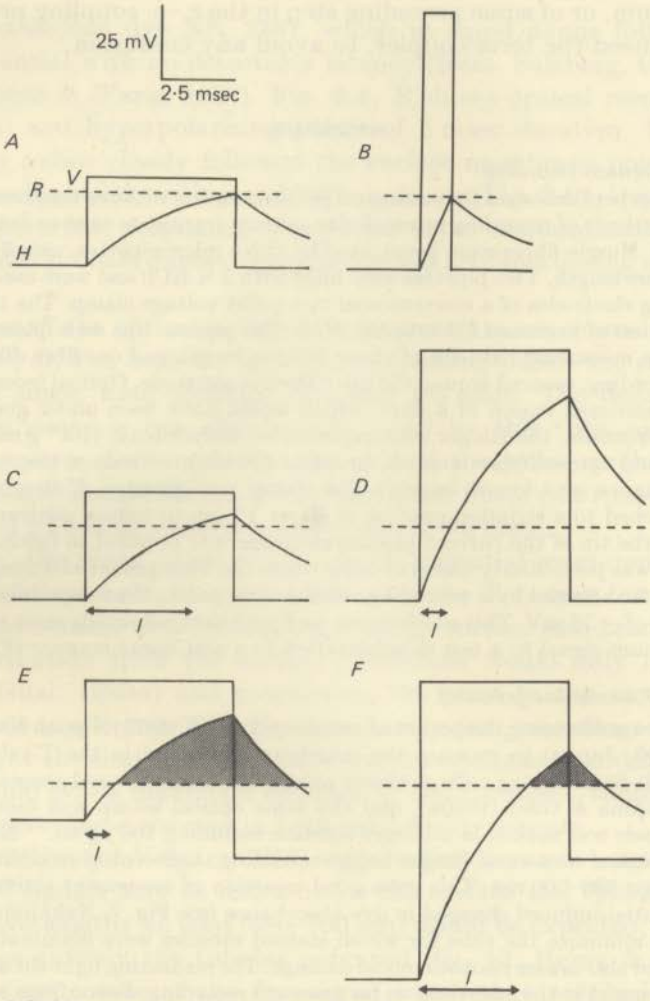


Fig. 1. A simplified linear model of e.–c. coupling. Depolarization of the surface membrane of a muscle fibre is presumed to lead to the build up of a hypothetical coupler. When the level of coupler exceeds a threshold value, release of calcium from the s.r. is triggered, at a rate which depends upon the amount by which the coupler exceeds the threshold. The threshold corresponds to a steady-state potential of −50 mV. The Figures illustrate how this model can explain the various observed properties of calcium release. In each Figure the rectangular pulses represent membrane potential, and the curved traces the time course of coupler build up and decay. Time constant for coupler build up and decay is 3 msec for this Figure. The dashed lines indicate the threshold coupler level for triggering calcium release. Potential levels marked *V*, *R* and *H* in (*A*) refer respectively to the test potential, rheobase potential and holding potential. *A*, *B*, threshold depolarizations to elicit a just detectable calcium release with different pulse durations. For brief durations (*B*), a larger depolarization is required before the coupler level reaches threshold. *C*, *D*, variation in latency of calcium release with depolarizing potential. As the depolarization is increased, the latency (*l*) before the coupler level crosses threshold decreases. *E*, *F*, variation in size of calcium response with different conditioning potentials preceding a test depolarization. The amount of calcium released by the pulse is proportional to the time integral of the coupler level above the threshold (indicated by shaded areas). When the fibre is hyperpolarized before a test pulse (*F*), this area is reduced. Note also that the latency before onset of calcium release (*l*) is increased at the more negative conditioning potential.

build up of calcium, or of some preceding step in the e.–c. coupling process. We have therefore introduced the term coupler, to avoid any confusion.

METHODS

Preparation and arsenazo recording

Experiments were performed on the cutaneous pectoris muscle of *Rana temporaria*. Details of the preparation and methods of recording intracellular calcium transients were as described previously (Miledi *et al.* 1982). Muscle fibres were penetrated by three micropipettes, usually positioned near the centre of the fibre length. Two pipettes were filled with 3 M-KCl, and were used as the recording and current passing electrodes of a conventional two-point voltage clamp. The third was used for ionophoretic injection of arsenazo III into the fibre. The pipette tips were placed within 100 μm of each other, and a measuring light slit of about 200 μm length and one fibre diameter width was used for optical recording, centred around the tip of the dye electrode. Optical records were therefore obtained from a restricted region of a fibre, which would have been under good voltage-clamp control. In all experiments, the Ringer solution included tetrodotoxin (10^{-6} g ml.$^{-1}$), to block the action potential, and tetraethylammonium bromide (20 mM), to reduce the voltage-dependent potassium conductance and hence improve the clamp performance. Unless otherwise stated, muscles were stretched to a striation spacing of about 3·7 μm to reduce contraction artifacts. In some experiments the tip of the current-passing electrode was bevelled to further improve clamp performance. This was particularly useful in cases where the fibre potential during a test pulse was otherwise found to be affected by a preceding conditioning pulse. Most experiments were made at a holding potential of −75 mV. This was because we found that potentials more negative than this influenced the calcium signal to a test depolarization in a non-linear manner (Fig. 11).

Optical recording of membrane potential

Experiments were made using the potential-sensitive dye NK 2367 (Nippon Kankoh-Shikiso Co. Ltd., Okayama 700, Japan) to monitor the membrane potential in the T-tubules and surface membrane of muscle fibres during voltage-clamp pulses. The methods used generally followed those described by Nakajima & Gilai (1980*a*), and the same optical set-up was used as for arsenazo recording. The muscle was soaked in a Ringer solution including 100 μg ml.$^{-1}$ NK 2367 for about 15 min, and was washed in normal Ringer before recording. Differential recordings were made at the wave-length pair 680–700 nm. This gave good rejection of movement artifacts and maximal sensitivity to potential-induced changed in dye absorbance (see Fig. 7, Nakajima & Gilai, 1980*a*). Care was taken to minimize the time for which stained muscles were illuminated, since the dye bleaches quickly and also causes photochemical damage. The measuring light slit and voltage-clamp electrodes were arranged in the same way as for arsenazo recording. Recordings were stored on FM tape (band width d.c. to 2·5 kHz), and were later averaged using a Data Labs DL 400B signal averager to improve the signal to noise ratio.

RESULTS

Optical measurement of T-tubule time constant

A possible complication in our measurements arises from the electrical time constant of the T-tubules, which might distort the time course of the voltage change sensed by the e.–c. coupling mechanism so that it no longer follows the rectangular voltage-clamp pulses. Results presented here, and previously (Miledi *et al.* 1981*b*; Adrian *et al.* 1969*a*), suggest that a time constant of a few milliseconds is involved in the e.–c. coupling process. It was therefore important to determine how much this could be accounted for by the T-tubule electrotonic time constant.

A direct method of investigating the T-tubule time constant is provided by the use of potential-sensitive dyes, which have been used to examine the propagation of the action potential along the T-tubules (Nakajima & Gilai, 1980*a*, *b*). We used the

merocyanine oxazolone dye NK 2367, which in squid axons follows changes in membrane potential with no detectable latency (Ross, Salzberg, Cohen, Grinvald, Davila, Waggoner & Wang, 1977). Fig. 2*A*, *B* shows optical records obtained in response to de- and hyperpolarizing pulses of 5 msec duration. The response to hyperpolarizing pulses closely followed the surface membrane potential, although with slight rounding of the rising and falling phases (Fig. 2*B*). Depolarizing pulses gave similar (inverted) responses, except for some contamination by a second component, which probably arose from mechanical artifacts during contraction (Fig. 2*A*).

The rise times of the optical potential measurements are shown on an expanded scale in Fig. 2*C*, *D*. For both de- and hyperpolarizing pulses the rising phases can be fitted by a single time constant of about 0·5 msec. The decay phase of the hyperpolarizing response can also be fitted well by this time constant, but the depolarizing response declines before the end of the pulse, probably because of movement artifact. Measurements from three other fibres (six runs) gave rise times between 0·5 and 0·75 msec, with a mean of about 0·6 msec.

Theoretically, the rising phase of the optical potential measurements would not be expected to follow a single exponential, since the T-system forms a distributed RC network, and because of contributions from the surface membrane. However, the expected contribution from the surface membrane would only be around 10% (Nakajima & Gilai, 1980*a*) and considering the noise in the recordings a single exponential gives an adequate fit to the data. In any case, recordings were made using a measuring light slit similar to that used during arsenazo recordings, so the value of 0·6 msec should give a maximum estimate of the average T-tubule time constant through the fibre volume covered in both sets of experiments.

A recent paper (Heiny & Vergara, 1982) suggests that the wave-length characteristics of the NK 2367 signals may be different for the surface and tubular membrane of muscle. The wave-lengths we used (680–700 nm) would be expected, from their data, to follow almost entirely the tubular potential (fig. 12, Heiny & Vergara, 1982). However, the time course of the dye signal which they ascribed to the tubular potential was much slower (several milliseconds) than in our records. We do not know the reason for this discrepancy; one possibility may be the use of a cut fibre preparation in the experiments of Heiny & Vergara.

Strength–duration curves for mechanical activation and calcium transient

The strength–duration curve for pulses which elicit a just detectable contraction provides an easily obtained measure of the kinetics of contractile activation, which has been used for several purposes (Adrian *et al.* 1969*a*; Almers & Best, 1976; Kovacs & Schneider, 1978). However, since this measurement includes all stages in e.–c. coupling from surface membrane depolarization to activation of the contractile filaments, interpretation is difficult. We were interested therefore to obtain the strength–duration curve for pulses required to elicit a just detectable rise in intracellular calcium, and to compare this with results using the conventional mechanical threshold.

Fibres were loaded with arsenazo III and the threshold pulse potential to elicit a just detectable calcium transient at 650–700 nm was measured for a range of pulse

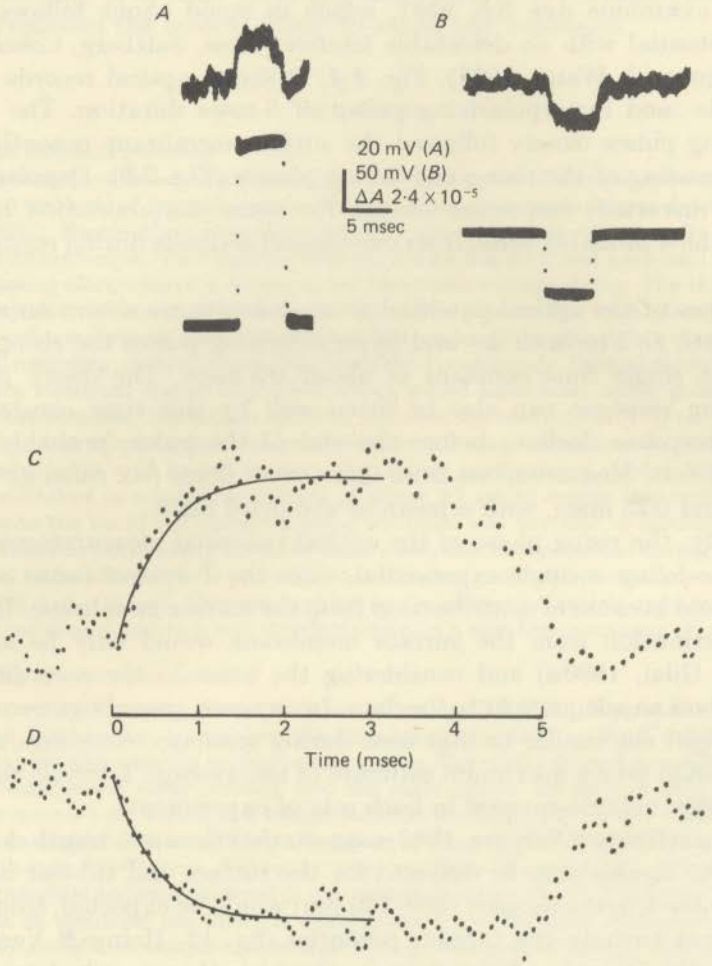


Fig. 2. Optical records of T-tubule membrane potential changes during voltage-clamp pulses, measured from fibres stained externally with NK 2367. Optical recordings were made at the wave-length pair 680–700 nm, with a band width of d.c. to 2 kHz. An upward deflexion indicates an increase in light absorbance at 680 nm, or a decrease at 700 nm. In this, and all subsequent optical records, the ΔA calibration gives the fractional change in light absorption; i.e. $\Delta A = \Delta I/2{\cdot}3$, where ΔI is the fractional change in transmitted light (Miledi *et al.* 1982). All records are signal averages of sixty-four sweeps, obtained at intervals of 0·5–2 sec. Temperature 9–11 °C. *A*, *B*, responses to respectively depolarizing and hyperpolarizing pulses of 5 msec duration. In each block the upper trace is the optical record and the lower trace the membrane potential of the fibre recorded through a micro-electrode. Note that the voltage calibration is different in (*A*) and (*B*). The depolarizing pulse was to a potential of +15 mV from a holding potential of −80 mV, and the hyperpolarizing pulse to −140 mV from −65 mV. *C*, *D*, enlarged tracings of averaged optical records to depolarizing (*C*) and hyperpolarizing (*D*) pulses, obtained from a different fibre to (*A*) and (*B*). The pulse duration is indicated by the time scale, and the rise and fall times of the voltage pulses applied to the fibre were about 0·2 msec (to 1/e). Rise time of the optical recording system (measured using pulses from a light emitting diode) was < 0·1 msec. No corrections for these rise times were made in estimating the T-tubule time constant. Curves fitted to the rising phases of the optical traces are exponentials with a time constant of 0·5 msec.

durations (Fig. 3*A*). The mechanical threshold was similarly determined, using a criterion of a just visible shortening of the sarcomeres when viewed through a microscope with ×400 magnification. Muscles were stretched less than usual, to a striation spacing of about 3 μm. Between pulses fibres were clamped at −75 mV.

Fig. 3*B* shows data from one fibre where complete sets of measurements of both calcium and mechanical thresholds were obtained. Both measurements lie closely together, indicating that under these conditions the strength–duration curve for

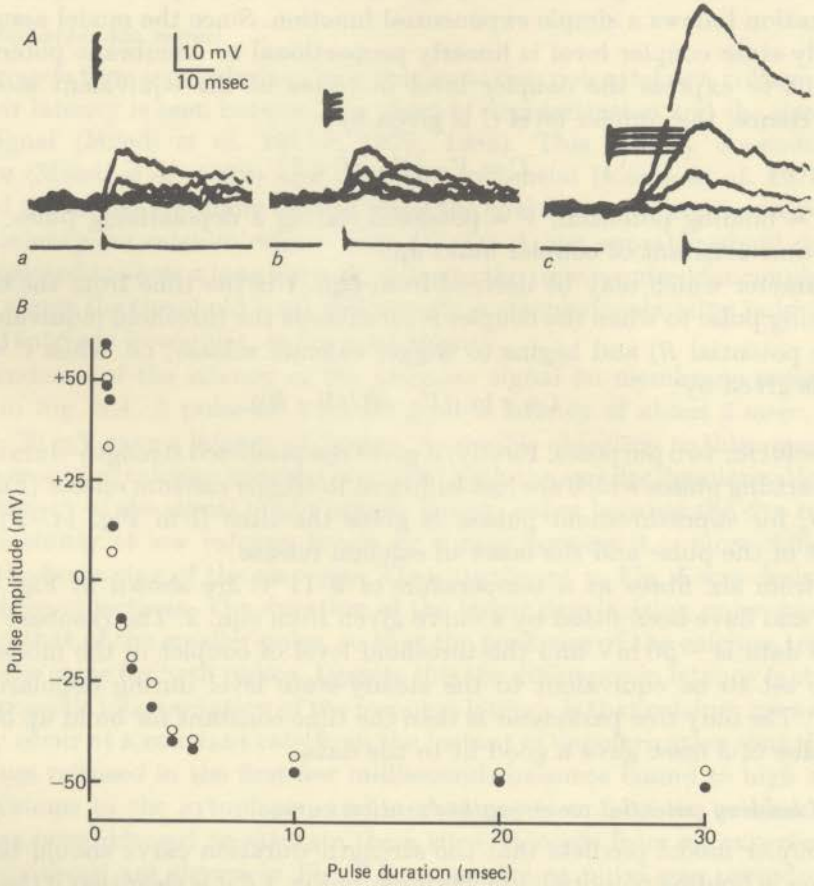


Fig. 3. Strength–duration relationship for threshold activation of calcium transient and mechanical contraction. *A*, records illustrating the method used to determine responses to stimulus pulses of different amplitudes. The lower trace in each frame is membrane potential, and the upper the arsenazo light absorbance signal recorded at the wave-length pair 650–700 nm. To determine the threshold potential for any particular pulse duration, a suprathreshold pulse was initially applied, and the pulse voltage was then reduced until stimulation just failed to elicit a response (smallest pulses in *a*–*c*). The pulse durations illustrated are 2 msec (*a*), 5 msec (*b*) and 20 msec (*c*). Pulse amplitudes were reduced in steps of 4 mV for (*a*) and 2 mV for (*b*) and (*c*). Threshold potentials could be determined to within about ±0·5 mV for long (> 10 msec) pulses, and to within about ±2 mV for pulses of 2 msec duration. *B*, strength–duration curves for threshold mechanical activation (open symbols) and calcium transient (filled symbols) measured from one fibre. The relationship was first measured using the arsenazo response, and then using the mechanical threshold.

mechanical activation reflects almost entirely the behaviour of some stage in e.–c. coupling prior to calcium release, and does not reflect the kinetics of calcium activation of the filaments. This observation is strengthened by similar results from four other fibres.

Quantitative description of strength–duration curve

For analysis of strength–duration curves for calcium activation we employed the predictions of the coupler model (Fig. 1). The build up of coupler during a depolarization follows a simple exponential function. Since the model assumes that the steady-state coupler level is linearly proportional to membrane potential, it is convenient to express the coupler level in terms of the equivalent steady-state voltage. Hence, the coupler level C is given by;

$$C = V-(V-H)\,e^{-t/\tau} \tag{1}$$

where H = holding potential, V = potential during a depolarizing pulse, t = time, and τ = time constant of coupler build up.

A parameter which may be derived from eqn. 1 is the time from the onset of a depolarizing pulse to when the coupler level exceeds the threshold (equivalent to the rheobase potential R) and begins to trigger calcium release; i.e. when $C = R$. This time (t) is given by;

$$t = \tau \ln ((V-H)/(V-R)). \tag{2}$$

This is useful for two purposes. Firstly, it gives the predicted strength–duration curve for depolarizing pulses which are just sufficient to trigger calcium release (Fig. 1 *A*, *B*). Secondly, for suprathreshold pulses, it gives the time (l in Fig. 1 *C–F*) between the onset of the pulse and the onset of calcium release.

Data from six fibres at a temperature of 9–11 °C are shown in Fig. 4 *A* (open squares) and have been fitted by a curve given from eqn. 2. The rheobase potential from the data is −50 mV and the threshold level of coupler in the model (R) was therefore set to be equivalent to the steady-state level during depolarization to −50 mV. The only free parameter is then the time constant for build up of coupler, and a value of 3 msec gave a good fit to the data.

Effects of holding potential on strength–duration curve

The coupler model predicts that the strength–duration curve should be affected by changes in holding potential. For example, in Fig. 1 *B* it is clear that if the potential preceding the depolarizing pulse were made more negative a greater depolarization would be required to achieve a threshold level of coupler. No changes in rheobase would be expected, however, since during a long pulse the coupler would approach the same value irrespective of the initial starting level. Results from experiments to test this hypothesis are shown in Fig. 4 *B*, where strength–duration curves were obtained at holding potentials of −60 and −100 mV. The results are qualitatively in agreement with the model prediction. At −60 mV the data are fitted well by a curve derived in the same way as for Fig. 4 *A*, with the parameters, rheobase = −48 mV and time constant = 3 msec (continuous curve in Fig. 4 *B*). At −100 mV the observed relationship is shifted in the expected way; there is no change in rheobase, but the threshold potential becomes more positive for briefer pulses.

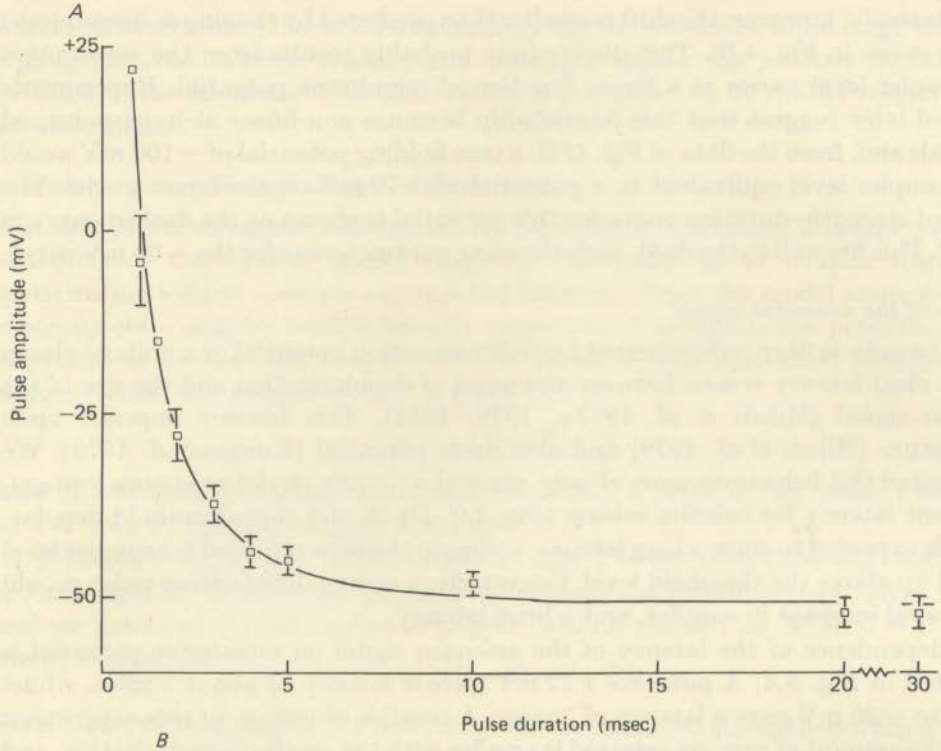
Quantitatively, however, the shift is smaller than predicted by the simple linear model (dotted curve in Fig. 4*B*). This discrepancy probably results from the assumption that coupler level varies as a linear function of membrane potential. Experiments described later suggest that this relationship becomes non-linear at hyperpolarized potentials and, from the data of Fig. 11*B*, a true holding potential of −100 mV would give a coupler level equivalent to a potential of −79 mV on the linear model. The predicted strength–duration curve for this potential is shown as the dashed curve in Fig. 4*B*. This fits well to the data, with the same parameters as for the −60 mV curve.

Latency of the arsenazo signal

When a muscle fibre is depolarized by either an action potential or a voltage-clamp pulse, a clear latency is seen between the onset of depolarization and the rise of the arsenazo signal (Miledi *et al.* 1977*a*, 1979, 1982). This latency depends upon temperature (Miledi *et al.* 1979) and also upon potential (Kovacs*et al.* 1979). We investigated this behaviour more closely, since the coupler model predicts a voltage-dependent latency for calcium release (Fig. 1*C*, *D*). A just suprathreshold depolarization is expected to show a long latency, owing to the time required for coupler level to build up above the threshold level. Conversely, a strong depolarizing pulse should give a rapid increase in coupler, and a brief latency.

The dependence of the latency of the arsenazo signal on membrane potential is illustrated in Fig. 5*A*. A pulse to +22 mV gave a latency of about 2 msec, whilst a pulse to −30 mV gave a latency of 5 msec. A possible objection to this experiment is that the amount of calcium released is smaller with the smaller depolarization, and hence the latency of the signal might appear longer, either because the dye reaction occurs more slowly at low calcium levels, or simply because it is more difficult to determine the beginning of the response. The experiment in Fig. 5 was designed to overcome these objections. The duration of the larger depolarizing pulse was made shorter than that of the smaller pulse, so that the peak size of the calcium transient was almost the same for both pulses. Despite this the difference in latency is striking.

Another possible interpretation of the response latency is that calcium release from the s.r. may occur at a constant rate from the instant of depolarization, but that the initial calcium released in the first few milliseconds becomes bound to high affinity buffering systems in the cytoplasm, and is thus not 'seen' by the arsenazo until sufficient has been released to saturate these sites. Records from an experiment to test this hypothesis are shown in Fig. 5*B*. A small test pulse was preceded by a conditioning pulse, which elicited a small, but clearly detectable, arsenazo signal. According to the hypothesis above, the calcium released during the conditioning pulse must have saturated any high affinity binding sites, since an arsenazo signal was seen. Hence, a large response would be expected to the test pulse, as all of the calcium released by this pulse would contribute to raising the free calcium level, rather than being partially bound to high affinity sites. In fact, the test response was found to be slightly smaller following the conditioning pulse (lower optical trace, Fig. 5*B*), than when applied alone (upper trace). Buffering of calcium ions is therefore probably not important in determining the latency of the arsenazo signal. The reduction in response size following the conditioning pulse probably resulted from a decreased calcium release from the s.r. (Miledi *et al.* 1983).



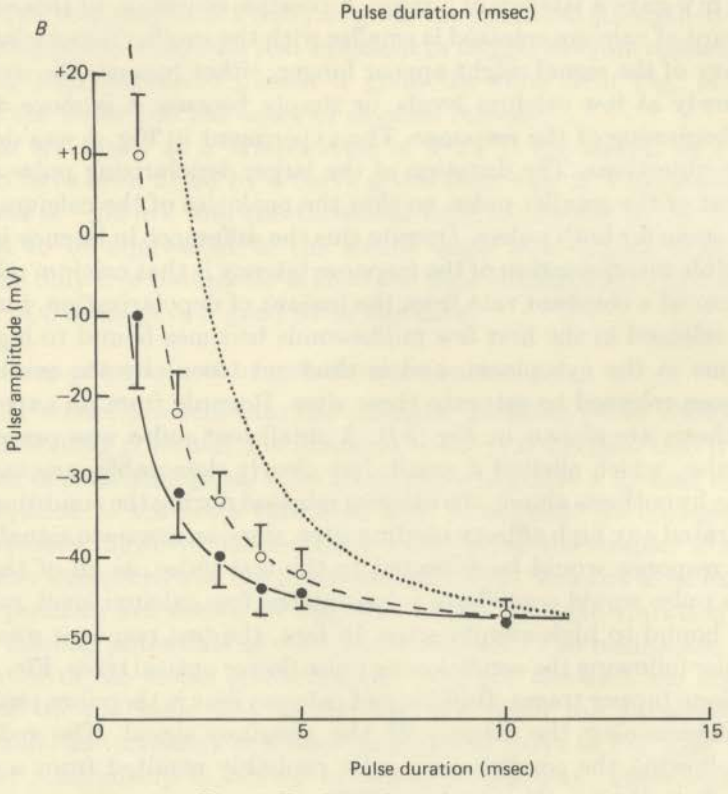


Fig. 4. For legend see facing page.

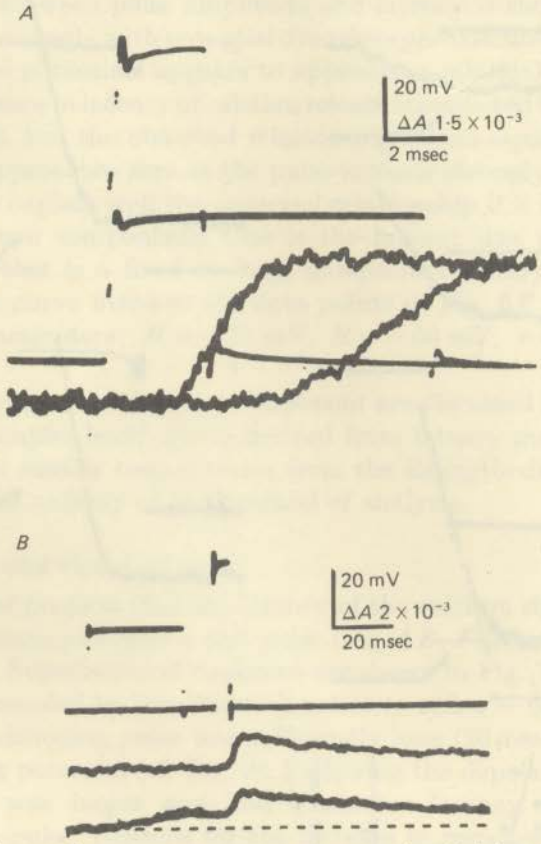


Fig. 5. *A*, superimposed arsenazo responses to two depolarizing pulses of different amplitudes and durations. Holding potential −75 mV. Upper traces show membrane potential. Optical record (lower traces) is light transmission at 650–700 nm, and was low-pass filtered at 2 kHz. *B*, arsenazo responses elicited by a 3 msec depolarizing test pulse alone (upper optical trace), or with a preceding depolarizing pulse (lower optical trace). A 10 msec interval was allowed between the conditioning and test pulses to avoid potentiation of the test response, but note that the calcium level had not returned to the base line at the time the test pulse was given. Temperature in (*A*) and (*B*), 9–11 °C.

Voltage dependence of signal latency

With depolarizations of increasing amplitude, the latency of the arsenazo signal decreases (Fig. 6*A–E*). The initial rate of rise of the signal was usually gradual, introducing some uncertainty in measurement of the latency. To circumvent this problem, we estimated the latency from the point at which the tangent to the main rising phase crossed the base line (Fig. 6*C*).

Fig. 4. *A*, Strength–duration curves for just detectable calcium signal at 10 °C. Error bars indicate ±1 S.E. of mean; points without bars are single observations. Data from six fibres. Curve was fitted as described in the text. *B*, strength–duration curves at different holding potentials. The membrane potential was clamped at −100 mV between test pulses for data shown by open symbols, and at −60 mV for filled symbols. Data from four fibres, each of which was examined at both potentials. Error bars are ±1 S.E. of mean. Curves were derived as described in the text. Temperature 9–11 °C.

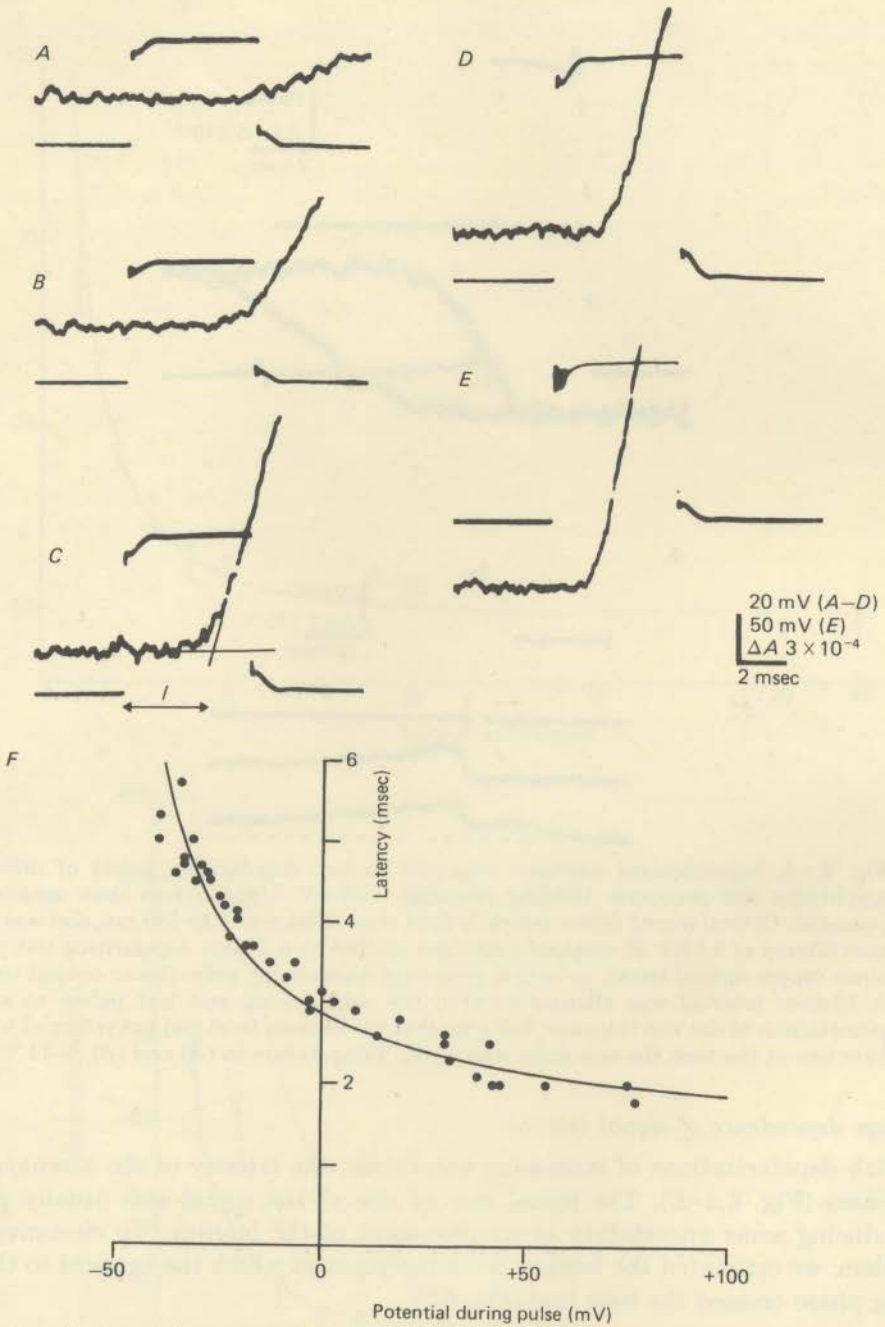


Fig. 6. *A–E*, change in latency of arsenazo response with depolarizing pulses of increasing amplitude. In each record the upper trace is membrane potential, and the lower, light transmission at 650–700 nm. All records from the same fibre. Optical trace low-pass filtered at 2 kHz. Temperature 10 °C. Note that the voltage gain was reduced in (*E*). Holding potential −75 mV in all records. Lines in (*C*) illustrate the method used to estimate the signal latency, *l*. *F*, relationship between the potential during a 5 msec duration depolarizing pulse and the latency to onset of the arsenazo signal. Data from four fibres. Temperature, 10 °C. Points are individual measurements, obtained as in (*C*). Holding potential, −75 mV. The curve was derived as described in the text.

The relationship between pulse amplitude and latency is shown in Fig. 6*F*. The latency varies most strongly with potential over the approximate range −40 to 0 mV, but at more positive potentials appears to approach a minimal limiting value.

A voltage dependence in latency of calcium release is predicted by the coupler model (Fig. 1*C*, *D*, eqn. 2), but the observed relationship differs significantly in that the predicted latency approaches zero as the pulse is made strongly positive. However, the model is able to explain well the observed relationship if it is supposed that the latency comprises two components. One is the latency due to coupler build up (eqn. 2), and the other is a fixed (voltage-independent) delay arising from some different cause. The curve fitted to the data points in Fig. 6*F* was derived on this basis, using the parameters; $H = -75$ mV, $R = -50$ mV, $\tau = 4$ msec and fixed latency = 1·2 msec.

Possible origins for the fixed latency component are discussed later. The estimated time constant for coupler build up (τ) derived from latency measurements is close to that estimated at similar temperatures from the strength–duration curves, thus giving support to the validity of this method of analysis.

Conditioning pulses and signal latency

The coupler model predicts that the latency of the calcium signal should increase if a fibre is hyperpolarized before a test pulse (Fig. 1*E*, *F*). Fig. 7 shows records to test this prediction. Superimposed responses are shown in Fig. 7*A* to a depolarizing pulse, which was preceded by conditioning pulses to either −60 or −110 mV. The duration of the conditioning pulse was sufficiently long (20 msec) to approximate a steady-state holding potential (cf. Fig. 9). Following the depolarizing pre-pulse, the arsenazo response was larger and had a shorter latency than following the hyperpolarizing pre-pulse. Reasons for the increase in response size are considered in the following section. The change in latency tends to be obscured by the amplitude changes, and to demonstrate more clearly the latency change we used the same stimulus pattern, but with the optical recording gain reduced during thedepolarizing pre-pulse records, so as to give final arsenazo traces of similar size. These records are shown in Fig. 7*B*, where there is a change in latency of about 1 msec due to the different pre-pulses.

The observed changes in arsenazo response latency therefore qualitatively bear out the prediction of the coupler model. We did not, however, make quantitative observations because of problems in measuring the small changes in latency.

Schneider, Rios & Kovăcs (1981) observed a similar shift in latency of the calcium signal caused by a subthreshold conditioning pulse, but they did not find any change in amplitude. This may be because they used a long and small (100 msec, −35 mV) test pulse, which would not be expected to show any large degree of potentiation by the conditioning pulse (Miledi *et al.* 1981*b*).

Potentiation and depression of response size by conditioning pre-pulses

The size of the arsenazo signal to a test depolarization is potentiated when preceded by a subthreshold depolarizing pulse (Miledi *et al.* 1981*a*, *b*; see also Fig. 7). This was interpreted in terms of the gradual build up of a potentiating process (Fig. 5, Miledi *et al.* 1981*b*), and on our present coupler model would be due to subthreshold build up of coupler during the pre-pulse (Fig. 1*E*, *F*). The degree of potentiation

produced by different durations of pre-pulse should therefore give a direct indication of the time course of build up of coupler, at least for potentials more negative than the rheobase. Experiments of this kind were reported previously (Miledi *et al.* 1981*b*), but the amount of data obtained was limited. In view of the importance of these measurements for the coupler model, we therefore obtained more extensive data.

Four types of experiment were performed. In the first, the depolarizing pre-pulse (to −60 mV from a holding potential of −75 mV) was given immediately before the

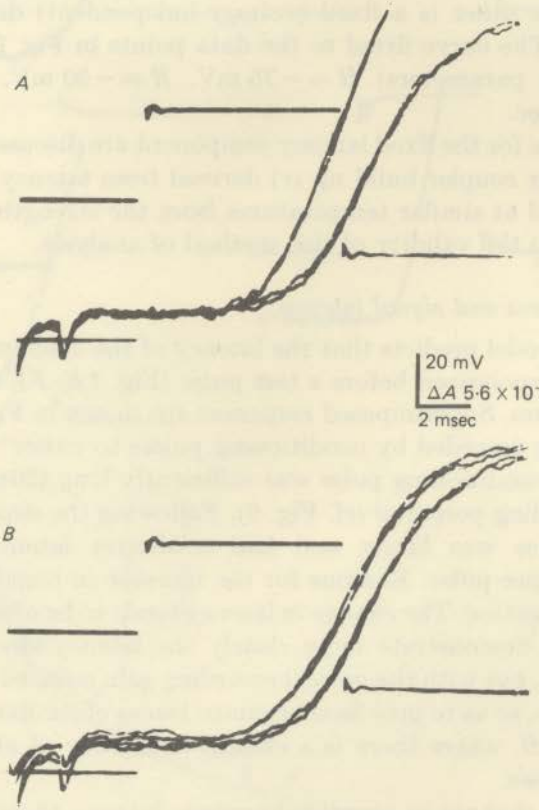


Fig. 7. Changes in latency of the arsenazo signal produced by depolarizing and hyperpolarizing pre-pulses. Each block shows superimposed responses to two stimuli with a depolarizing pre-pulse, and two sweeps with a hyperpolarizing pre-pulse. Upper traces are membrane potential, and lower (noisy) traces are light transmission at 650–700 nm, low-pass filtered at 2 kHz. Holding potential was −75 mV, and records return to this potential at the end of test pulses. Duration of the pre-pulses was 20 msec; the pulses begin before the start of the records. Downward blips at the start of the optical records are artifacts from the reset circuit. The voltage-clamp control was particularly good in this experiment, and the potential during the test pulse was not noticably altered by the different pre-pulse potentials. Temperature 10 °C. *A*, superimposed records from a fibre using a fixed optical recording gain. The more rapidly rising responses were elicited following the depolarizing pre-pulse. *B*, similar records from the same fibre, but with the recording gain on the oscilloscope reduced whilst recording with depolarizing pre-pulses, so as to give similar apparent response sizes for the two conditioning voltages. Vertical scale for the optical traces is uncalibrated for this record. The shorter latency responses were elicited following the depolarizing pre-pulse.

test pulse, and was of variable duration (Fig. 8*D–F*). This gives information on the time course of build up of potentiation. In the second experiment, the pre-pulse duration was fixed at 10 msec, but the interval between the end of the conditioning pulse and the start of the test pulse was variable (Fig. 8*A–C*). This gives information on the time course of decay of potentiation. Additionally, we found that hyperpolarizing pre-pulses (from a holding potential of −75 mV) cause a depression of the arsenazo signal to a test depolarization. Similar experiments were carried out also using hyperpolarizing pre-pulses, to investigate the time course of build up and decay of depression.

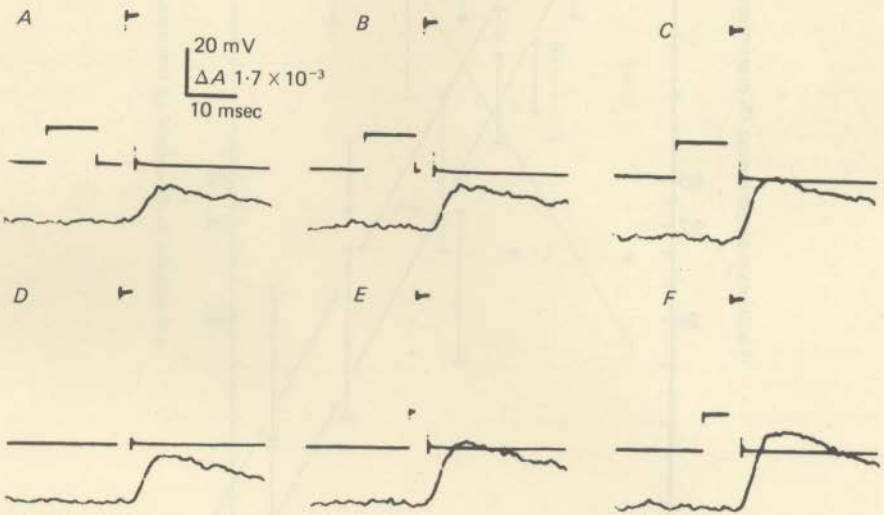


Fig. 8. Records illustrating the potentiation of the arsenazo signal to a test depolarization, due to conditioning pre-pulses. In each block the upper trace is membrane potential, and the lower, light transmission at 650–700 nm. Sixty second intervals were allowed between each test to allow the response to recover to the rested state level. Holding potential was −75 mV, test pulse duration 3 msec, and conditioning pulse potential −60 mV. Temperature 10 °C. *A–C*, a fixed duration of conditioning pulse was used (10 msec), and the interval between the end of the conditioning pulse and the start of the test pulse was varied. *D–F*, the duration of the conditioning pulse was varied, with no gap before the start of the test pulse. Records in *D–F* were obtained from the same fibre as *A–C*, but after an interval of about 10 min. The response size to similar stimulating pulses was larger at this time (e.g. compare *C* and *F*), probably because the intracellular dye concentration had risen.

Brief (3 msec) and small (−10 mV) test pulses were used, so as to enhance the extent of potentiation or depression (Miledi *et al.* 1981*b*). A problem was that the potential during the test pulse tended to be affected by the preceding conditioning pulse, probably because of changes in the current electrode. In general, we attempted to achieve sufficiently good clamp control for this not to be a problem (< 1 mV change in pulse amplitude), but for a few fibres a correction was applied as follows; at the beginning of a run arsenazo responses were elicited by test pulses covering a range of amplitudes around 10 % of the control value. The relationship between pulse size and response was plotted, and used to correct other readings where the pulse size was affected by a conditioning pulse.

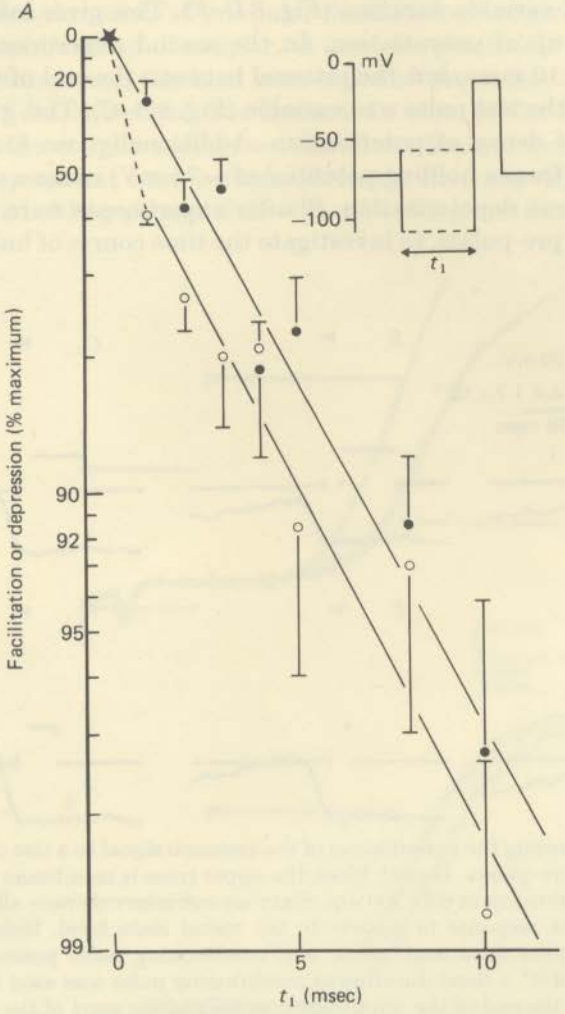


Fig. 9. For legend see facing page.

Fig. 9 shows the time courses of build up and decay of potentiation and depression. The data are on a semi-logarithmic scale, and the ordinate indicates the degree of potentiation or depression as a percentage of the maximum for each fibre. This value was obtained by first expressing each response size with a pre-pulse as a percentage of the control response without pre-pulse. The percentage potentiation or depression was then calculated as a percentage of the maximum for that fibre, following a 20 msec duration pre-pulse with no gap between pre- and test pulses. This allowed data to be pooled from several fibres showing different extents of maximial potentiation and depression.

Fig. 9 *A* gives the time courses of build up of potentiation and depression, measured using different durations of pre-pulse. Both fitted well to a single exponential over most of the range, with a time constant of about 3 msec. The depression however appears to comprise two components, since very short (< 1 msec) pre-pulses produce

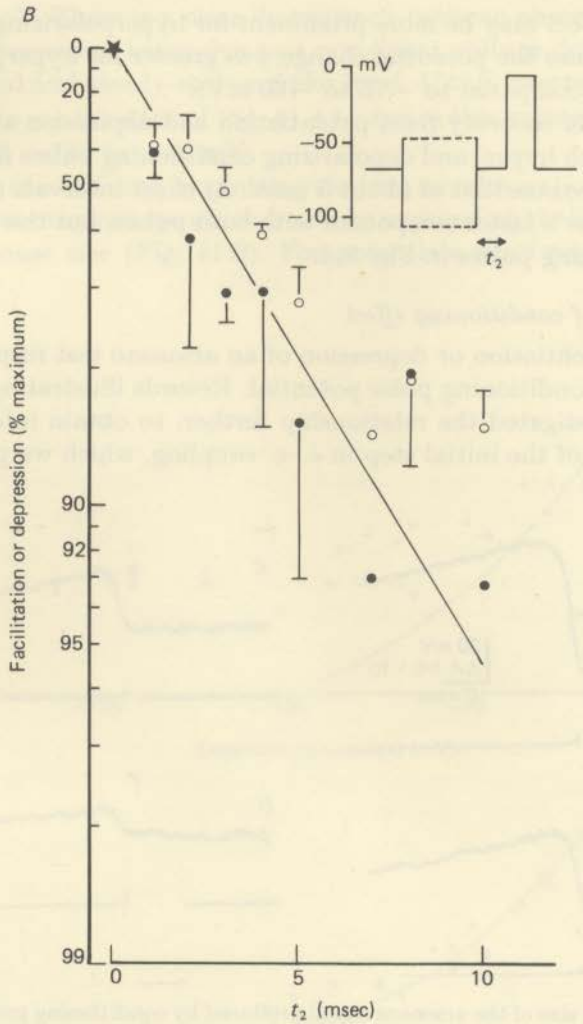


Fig. 9. Time courses of potentiation and depression, plotted on semi-logarithmic co-ordinates. In each graph, data are shown for depolarizing pre-pulses to −60 mV (filled symbols) and hyperpolarizing pre-pulses to −110 mV (open symbols). Holding potential was −75 mV, and test pulse potential about −15 mV. Insets illustrate the pulse protocol. Graph ordinates give the percentage of maximum potentiation or depression, as described in the text. Bars indicate ±1 S.E. of mean. Temperature 9–11 °C. *A*, development of potentiation and depression, plotted against duration of the pre-pulse (t_1). Data from six fibres. Lines were fitted by eye. Continuous lines correspond to a time constant of 2·8 msec. *B*, recovery from potentiation and depression, plotted against the length of the gap (t_2) between the conditioning pulse and the test pulse. Conditioning pulse duration was 10 msec. Data from seven fibres. Line fitted by eye, with a time constant of 5 msec.

more depression than expected from a single time constant of 3 msec (see also Miledi *et al.* 1981*b*). This was investigated at finer resolution in one experiment (three fibres), which indicated that the initial build up of depression has a time constant of about 0·6 msec. Electrotonic charging of the T-system may explain this fast component, and the time constant is in agreement with that obtained using potential-sensitive

dye (Fig. 2). This effect may be more prominent for hyperpolarizing pulses than for depolarization, because the potential change was greater for hyperpolarizing pulses (-75 to -110 mV, compared to -75 to -60 mV).

The time courses of recovery from potentiation and depression are shown in Fig. 9*B*. The data for both hyper- and depolarizing conditioning pulses fit well over most of the range to a time constant of about 5 msec. At short intervals (< 1 msec) there was some evidence for a faster component with both pulses, but this was not as clear as with hyperpolarizing pulses in Fig. 9*A*.

Voltage dependence of conditioning effect

The extent of potentiation or depression of an arsenazo test response varies as a graded function of conditioning pulse potential. Records illustrating this are shown in Fig. 10. We investigated the relationship further, to obtain information on the voltage dependence of the initial step in e.–c. coupling, which we presume leads to build up of coupler.

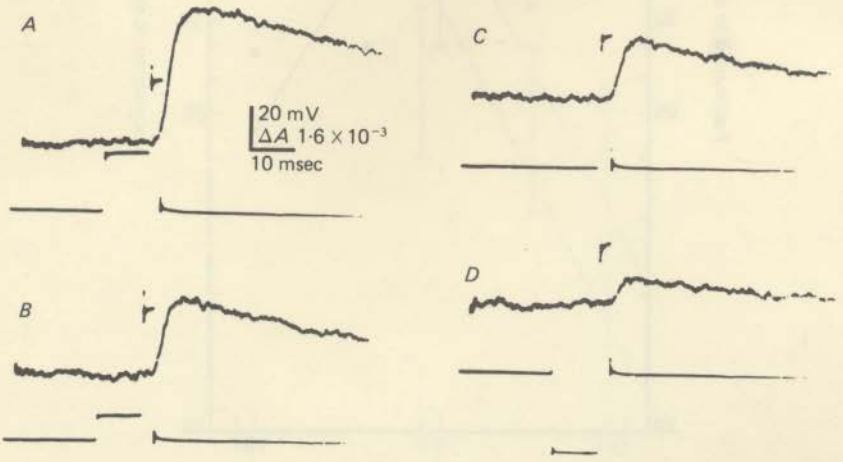


Fig. 10. Changes in size of the arsenazo signal produced by conditioning pulses of different amplitudes. Results from one fibre. Records *A*–*D* show the effects of conditioning voltages between -60 and -110 mV. In each block, the upper trace is the arsenazo signal, and the lower membrane potential. Test pulse duration was 3 msec, and amplitude -15 mV. Conditioning pulse duration, 10 msec. Temperature, 10 °C.

The relationship between conditioning potential and response size measured from three fibres is shown in Fig. 11*A*. Measurements were made as in Fig. 10, and were expressed as a percentage of the response size when preceded by a conditioning pulse to -50 mV (this conditioning pulse was just threshold for a detectable calcium signal). As the conditioning pulse is made more negative the response size decreases, but reaches a minimal value (about 25% of the -50 mV response) and does not decrease further with hyperpolarization beyond about -100 mV.

The linear coupler model (eqn. 1 and Fig. 1*E*, *F*) predicts that the response should decrease with increasingly negative conditioning pulses, and should become zero at strongly negative potentials. The curve in Fig. 11*A* shows the predicted model behaviour for the following parameters; $\tau = 3$ msec, $R = -50$ mV, test pulse duration

3 msec, $V = -15$ mV. There is a clear discrepancy between observed and predicted data, which we interpret as being due to a non-linear voltage dependence between membrane potential and steady-state coupler level. If this interpretation is correct, then it is possible to use the data of Fig. 11*A* to derive this voltage dependence over the potential range -140 to -50 mV.

The relationship can be found by plotting the true membrane potential against the steady-state conditioning voltage which would be required on the linear model to give the observed response size (Fig. 11*B*). For potentials more negative than about

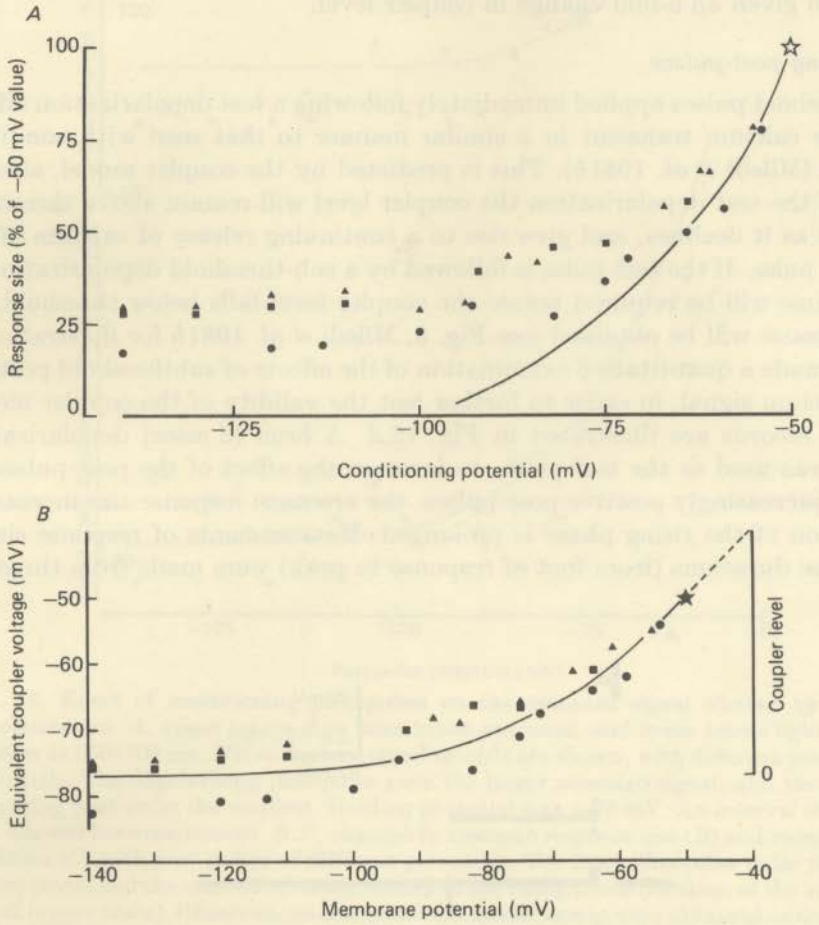


Fig. 11. *A*, relationship between conditioning pulse voltage and size of arsenazo response. Data were obtained from records similar to those in Fig. 10, using the same pulse parameters. Results from three fibres are indicated by different symbols. Temperature, 9–11 °C. The response size is expressed as a percentage of the mean value for each fibre at a conditioning potential of -50 mV (indicated by star). The curve shows the predicted behaviour of the linear coupler model for the parameters given in the text. *B*, estimated voltage dependence of coupler activation derived from the data in (*A*). See text for details. Left-hand ordinate gives the steady-state potentials predicted by the linear coupler model which would be required to give the observed response sizes following conditioning pulses to the true membrane potentials indicated on the abscissa. Right-hand ordinate illustrates the presumed coupler level. Symbols correspond with those in (*A*). Curve was drawn by eye.

−100 mV the relationship is flat, whilst at potentials more positive than about −60 mV it approaches linear, but because of the necessity to use subthreshold conditioning voltages, only a restricted range (up to −50 mV) could be examined. However, the fact that the linear coupler model describes well the observed strength–duration relationship for depolarizations up to positive potentials (Fig. 4*A*) suggests that a linear relationship holds between −60 and (at least) 0 mV. At potentials close to threshold for coupler activation (−100 mV) the data in Fig. 11*B* approximate to an exponential function, with a potential change of about 14 mV required to given an e-fold change in coupler level.

Conditioning post-pulses

Subthreshold pulses applied immediately following a test depolarization affect the size of the calcium transient in a similar manner to that seen with conditioning pre-pulses (Miledi *et al.* 1981*b*). This is predicted by the coupler model, since after the end of the test depolarization the coupler level will remain above threshold for some time as it declines, and give rise to a continuing release of calcium after the end of the pulse. If the test pulse is followed by a sub-threshold depolarization, then a longer time will be required before the coupler level falls below threshold, and a larger response will be obtained (see Fig. 5, Miledi *et al.* 1981*b* for illustration). We therefore made a quantitative examination of the effects of subthreshold post-pulses on the calcium signal, in order to further test the validity of the coupler model.

Typical records are illustrated in Fig. 12*A*. A brief (3 msec) depolarization to −15 mV was used as the test pulse, to increase the effect of the post-pulses. Note that with increasingly positive post-pulses, the arsenazo response size increases and the duration of the rising phase is prolonged. Measurements of response sizes and rising phase durations (from foot of response to peak) were made from three fibres,

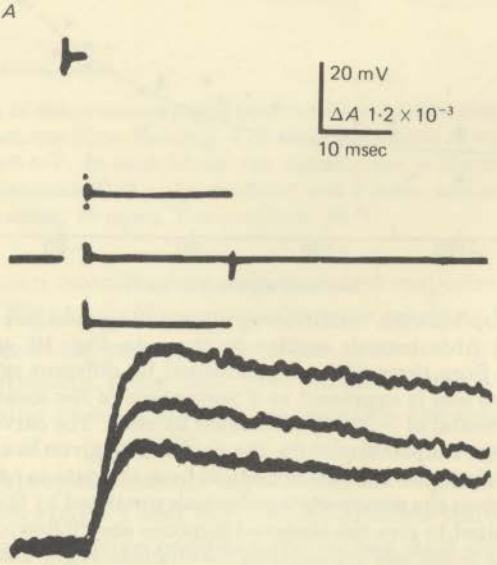


Fig. 12. For legend see facing page.

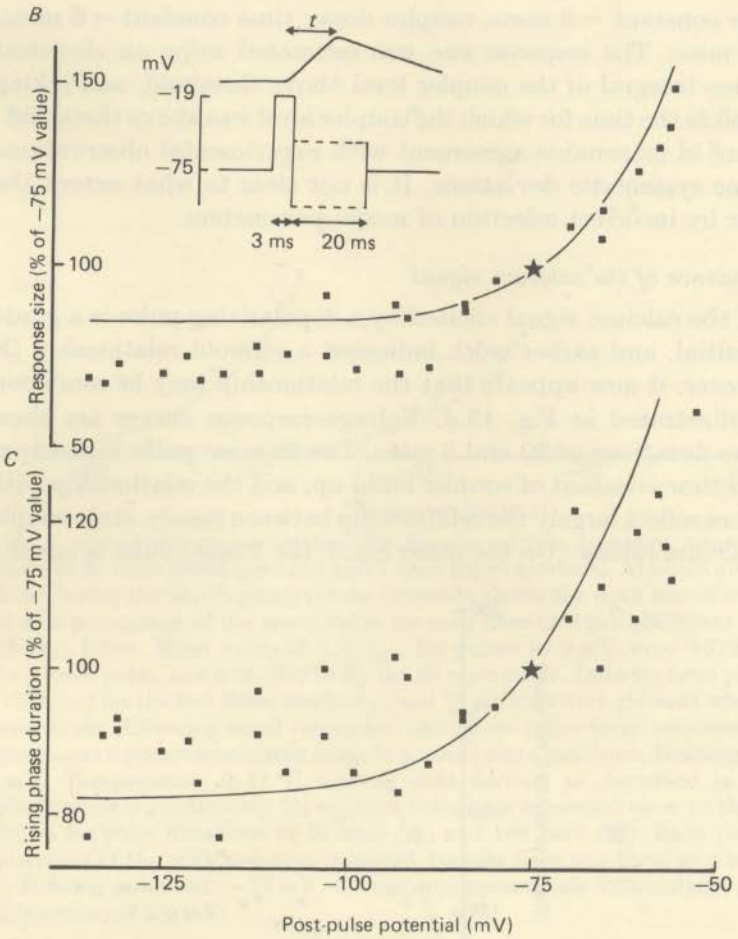


Fig. 12. Effect of conditioning post-pulses on the arsenazo signal elicited by a test depolarization. *A*, upper traces show membrane potential, and lower traces light transmission at 650–700 nm. Three superimposed records are shown, with different post-pulse potentials. The depolarizing post-pulse gave the larger arsenazo signal, and the hyperpolarizing post-pulse the smallest. Holding potential was −75 mV. An interval of 60 sec was allowed between stimuli. *B*, *C*, changes in arsenazo response size (*B*) and rising phase duration (*C*) with post-pulses of different potentials. The inset illustrates pulse protocol (lower trace) and the method of measurement of the rising phase duration of the arsenazo signal (upper trace). Observations and predicted model curves were obtained as described in the text. Data from three fibres at 10 °C.

using different post-pulse voltages in a similar way as for Fig. 11*A*. These are shown in Fig. 12*B*, *C*, expressed as a percentage of the value with no post-pulse (i.e. at a holding potential of −75 mV). Both the response size and rising phase duration increase as the post-pulse potential is made more positive than about −90 mV, but show little voltage dependence at potentials more negative than this.

The predicted behaviour of the non-linear coupler model is indicated by the curves (Fig. 12*B*, *C*). These were derived from the linear model, but using the data of Fig. 11*B* to transform from post-pulse membrane potential to equivalent steady-state coupler voltage. Parameters were; $H = -75$ mV, $V = -15$ mV, $R = -50$ mV, coupler

build up time constant = 3 msec, coupler decay time constant = 5 msec, test pulse duration = 3 msec. The response size was estimated using an electrical circuit to obtain the time integral of the coupler level above threshold, and taking the rising phase duration as the time for which the coupler level was above threshold. The model predictions are in reasonable agreement with experimental observations, although there are some systematic deviations. It is not clear to what extent these may be accounted for by incorrect selection of model parameters.

Voltage dependence of the calcium signal

The size of the calcium signal elicited by a depolarizing pulse is a graded function of pulse potential, and earlier work indicated a sigmoid relationship (Miledi *et al.* 1977*a*). However, it now appears that the relationship may be more complex than this, and is illustrated in Fig. 13*A*. Voltage–response curves are shown for two different pulse durations of 20 and 3 msec. The 20 msec pulse is nearly seven times the estimated time constant of coupler build up, and the relationship with this pulse might therefore reflect largely the relationship between steady-state coupler level and the rate of calcium release. On the other hand, the 3 msec pulse is equal to only one

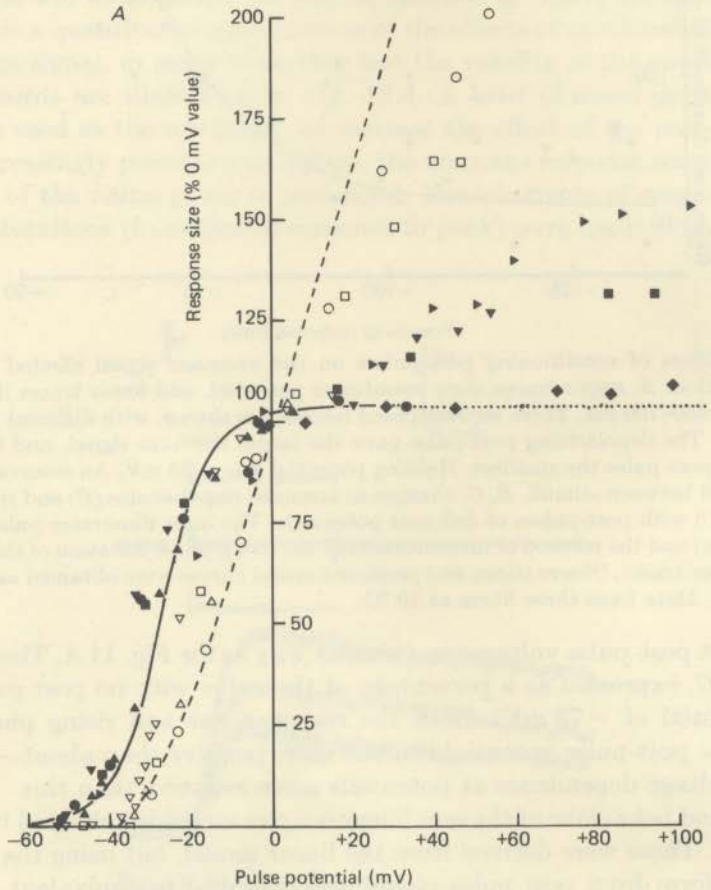


Fig. 13. For legend see facing page.

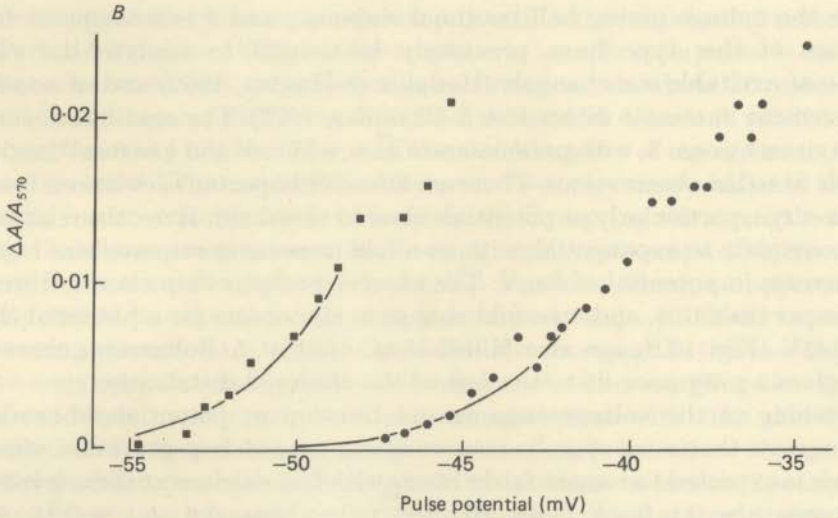


Fig. 13. *A*, stimulus–response relationship for the calcium transient measured for pulse durations of 20 msec (filled symbols) and 3 msec (open symbols). Abscissa gives membrane potential during the depolarizing pulses. Ordinate shows the peak size of arsenazo signal scaled as a percentage of the mean value for each fibre at 0 mV. Different symbols refer to different fibres. Mean value of $\Delta A/A_{570}$ for pulses to 0 mV were $0{\cdot}072 \pm 0{\cdot}034$ (S.D.) for the 3 msec pulse, and $0{\cdot}24 \pm 0{\cdot}075$ for the 20 msec pulse. Data for both pulse durations were obtained for the two fibres marked △ and ▽. Stimuli were given at intervals varying between 30 sec (following small responses) and 80 sec (after large responses), so that all measurements were obtained from fibres in a rested state condition. Holding potential was −75 mV. Temperature, 9–11 °C. Curves were derived as described in the text. *B*, stimulus–response relationship for calcium transients measured close to threshold. Data are shown for pulse durations of 10 msec (●) and 100 msec (■). Each point is a single measurement of the peak arsenazo response. Results from one fibre, at a temperature of 10 °C. Holding potential, −75 mV. Curves are exponentials with voltage constants (for e-fold increase) of 2·8 mV.

time constant of coupler build up (at 10 °C), and so the voltage–response relationship is expected to be considerably modified by the kinetics of coupler build up and decay. Pulse durations of 5 and 10 msec were also examined, and gave relationships intermediate between those with 3 and 20 msec pulses.

Considering first the relationship with the 20 msec pulse (Fig. 13*A*), the threshold potential for a detectable calcium signal is about −55 mV, and the response then rises steeply with potential up to about −10 mV. At more positive potentials considerable variation was observed between different fibres; some (marked ◆ and ● in Fig. 13*A*) showed no change in response size with further depolarization, whilst others showed a gradual increase (fibres marked ▶, ▼ and ■). Voltage decrements along the T-tubules might at least partially account for the gradual increase seen in some fibres, although the potential at the centre of a 100 μm diameter fibre would be expected to be at most only 10–15 mV lower than near the surface (Adrian *et al.* 1969*b*). Excepting the gradual increase at positive potentials, the data appear to follow a sigmoid curve. This may be expressed empirically by a Boltzmann distribution, where the response size R at any given voltage V is given by:

$$R = R_{\max}/(1+\exp(-(V-\overline{V})/k)) \qquad (3)$$

where $\overline{V}$ is the voltage giving half maximal response, and k is a steepness factor. Distributions of this type have previously been used to analyse the voltage dependence of excitable ion channels (Hodgkin & Huxley, 1952) and of non-linear charge movement in muscle (Schneider & Chandler, 1973). The continuous curve in Fig. 13*A* is given by eqn. 3, with parameters of $\overline{V} = -27$ mV and $k = 6$ mV, and gives a reasonable fit to the observations. There are however important deviations from the Boltzmann curve, particularly at potentials close to threshold. Here, the relationship should approximate to exponential, with an e-fold increase in response size resulting from an increase in potential of k mV. The observed relationship close to threshold is much steeper than this, and an e-fold change in size occurs for a potential change of about 3 mV (Fig. 13*B*; see also Miledi *et al.* 1981*b*). A Boltzmann curve with $k = 3$ mV gives a very poor fit to the rest of the observed distribution.

The flattening of the voltage–response relationship at potentials above about −20 mV suggests that some stage in e.–c. coupling approaches saturation, since the dye response is expected to remain fairly linear with free calcium at these levels. The mean response size at 0 mV with 20 msec pulses was $\Delta A/A_{570} = 0{\cdot}24$, which indicates that just over one-third of the dye was binding calcium (saturating value of $\Delta A/A_{570} = 0{\cdot}615$; Miledi *et al.* 1982). In agreement with this, larger arsenazo signals could be obtained using pulse durations longer than 20 msec. On the basis of the coupler model, saturation of the response could occur from either saturation of the relationship between potential and coupler level, or from saturation of the coupler level–calcium release relationship. Observations with a stimulus pulse duration of 3 msec (Fig. 13*A*) suggest that it is the latter which is the more important. If the voltage-coupler level relation were saturating, then the observed relationship would be expected to level off above about −20 mV, in a similar way to the 20 msec pulse data. However, this is not seen, and instead the calcium signal continues to increase steeply for potentials up to at least +50 mV.

The difference in the stimulus–response relationship seen with pulse durations of 3 and 20 msec can, to a large extent, be accounted for by the coupler model. The dashed line in Fig. 13*A* shows the prediction of the model, using a simplifying assumption that calcium release is linearly proportional to coupler level for potentials between −50 mV and −10 mV, and is, respectively, zero for more negative potentials, and 100% for more positive potentials. Integration of the coupler level (as in Fig. 1*E*) between these limits was used to obtain the curve, which was scaled to pass through the 0 mV data points. For negative potentials the predicted curve fits well to the data, but at positive potentials the observations are smaller than predicted. This may suggest that the relationship between potential and coupler level also begins to saturate at positive potentials.

DISCUSSION

Linearity of arsenazo recordings

Calcium release from the s.r. into the myoplasm was monitored using arsenazo III, an indicator dye which is highly specific for calcium under the conditions used (Miledi *et al.* 1982). Some results involved measurements of changes in size of arsenazo signals under different conditions, and for analysis we assumed a linear relationship between signal size and amount of calcium released. This linearity has been directly demon-

strated for ionophoretic injection of calcium pulses into arsenazo-loaded muscle fibres (Miledi, Parker & Schalow, 1980), but the response to ionophoretic pulses is slow, while here we are concerned with response peaks occurring within about 20 msec. Recent work (Palade & Vergara, 1982) has indicated that under transient conditions, the arsenazo response may not be linear, and the stoichiometry of the arsenazo reaction is still controversial (Thomas, 1982). However, two arguments indicate that any non-linearity in our experiments is not serious. (i) Excepting the strength–response curve (Fig. 13), all measurements were made using small responses ($< 10\%$ maximum). Thus, even if the over-all response relationship were non-linear, an approximation to linearity would be expected over such a restricted range. (ii) Observations were made also using arsenazo to detect the threshold calcium signal. In this case the nature of the dye response relationship is immaterial, but the e.–c. coupling time constant derived from these experiments was in good agreement with that derived from experiments involving quantitative measurement of response sizes.

A related problem is whether the free calcium changes in muscle linearly reflect the amount of calcium released from the s.r. Uptake of calcium from the myoplasm can be neglected for the present purposes, since the time to peak of the transients is appreciably faster than the decay time of the response (10–20 msec, compared to 80 msec; Miledi *et al.* 1982). The myoplasm probably contains a high concentration of calcium buffering sites such that for physiological levels of calcium release the free calcium concentration is much lower than, but approximately linearly proportional to, the total amount of released calcium (Miledi *et al.* 1982). However, it remained possible that the presence of a low concentration of high affinity binding sites could introduce an apparent delay in onset of the arsenazo response. The experiment of Fig. 5*B* indicates that this is not the case.

Electrical properties of the T-tubules

E.–c. coupling is triggered by a potential change across the T-tubule membrane, and the electrical time constant of the tubular system will distort this potential relative to pulses applied across the surface membrane. If this time constant were sufficiently long (3–4 msec at 10 °C) it would account substantially for our observations of the time-dependent process in e.–c. coupling. Several arguments suggest that this is not the case, although the available data are not entirely conclusive.

(i) As discussed previously (Miledi *et al.* 1981), the time constant of the T-system charging process from the Falk & Fatt model (1964) is reported to be 0·6 msec (3–6 °C; τ_s in Table 3 of Adrian *et al.* 1970). This is appreciably faster than our estimate of coupler build up (3–4 msec at 10 °C). Against this, however, the data of Adrian *et al.* (1969*b*) indicate that the ratio of depolarizations required to activate axial myofibrils to that causing contraction of superficial myofibrils was consistently less for 3 msec than for longer pulses (at 20 °C).

(ii) Direct recording of the over-all tubular membrane potential using the potential-sensitive dye NK 2367 indicates that the potential follows a step with a time constant of about 0·6 msec (at 10 °C).

(iii) The coupling process shows a non-linear voltage dependence, in that conditioning hyperpolarizations more negative than about -90 mV give no further reduction in calcium signal to a test depolarization (Fig. 11*A*). This non-linearity

would not be expected if the passive electrical properties of the T-system were responsible for the potentiation and depression seen with conditioning pulses. It may be noted that this argument is essentially the same as used in interpretation of charge movement data in muscle (Schneider, 1981; Adrian, 1978).

A related problem is the decrement in potential along the T-tubules, which is expected even in the steady state (Adrian *et al.* 1969*b*). Errors from this source should be quite small (e.g. less than about 10 % at the centre of a 100 μm diameter fibre from Fig. 1, Adrian *et al.* (1969*b*)), and we have not attempted to apply any correction for this factor. In the case of depolarizations close to threshold at least, no correction should be required, since all the calcium release presumably occurs from superficial regions of the fibre.

Strength-duration curves

The finding that strength–duration curves measured for threshold activation of the calcium signal or of contraction are closely similar is interesting, since it means that the strength–duration curve for contraction accurately reflects the e.–c. coupling process leading to calcium release, and is not distorted by the characteristics of calcium binding to troponin and subsequent contractile activation. Strength–duration curves for contractile activation have been measured many times in the past (e.g. Adrian *et al.* 1969*a*; Almers & Best, 1976; Horowicz & Schneider, 1981*b*) and have generally been interpreted as reflecting the characteristics of e.–c. coupling, although until recently (Horowicz & Schneider, 1981*b*) with little direct evidence for this assumption. One important feature of our results is that the rheobase potential measured with relatively long pulses (e.g. 20 msec at 10 °C) is still determined principally by the calcium release system, rather than reflecting a balance between calcium release and re-uptake by the s.r. The time constant of decline of the calcium transient is quite slow compared to this pulse duration (80 msec following an action potential at 10 °C; Miledi *et al.* 1982), so that uptake of calcium would have only a slight influence on strength–duration curves.

The coupler model of e.–c. coupling

Our major aims in these experiments were to investigate the voltage- and time-dependent characteristics of calcium release from the s.r., and to provide a simple quantitative account of these characteristics. We have interpreted the results in terms of a model in which depolarization leads to the build up of a hypothetical coupler, which in turn triggers calcium release when a threshold is exceeded. This model does not require any assumptions as to the physical basis of the coupler. One possibility is that it represents the electrical charging of the T-system time constant. However, as discussed earlier, we feel that this explanation is unlikely, and instead believe that the coupler most probably represents an intermediate stage between tubular depolarization and calcium release from the s.r.

The model comprises three distinct stages: (1) voltage-dependent activation of coupler build up; (2) time-dependent changes in coupler level and (3) activation of calcium release. Experimental observations of calcium signals are described well by the model, specifically: (i) the strength–duration curve for threshold calcium release; (ii) the effects of subthreshold pulses applied before or after a test depolarization and

(iii) the voltage-dependent latency of onset of the calcium signal. The time constant of coupler build up was directly estimated from the potentiation of a test signal by subthreshold conditioning depolarizations of different durations, and gave a value of 3 msec at 10 °C. Similar estimates were also obtained from the measurements in (i)–(iii) above.

An interesting feature is that the time constant of coupler build up does not appear to vary appreciably with membrane potential. Direct measurements of the build up of potentiation and depression produced by conditioning pulses (which corresponds to the coupler build up and decay on the model) were obtained at potentials of −110 and −60 mV, and gave similar values. It is likely also that at more positive potentials the time constant shows little change, since we were able to fit the strength–duration and latency data on the assumption of a fixed time constant. Our model differs, therefore, from the similar 'activator' model of Adrian *et al.* (1969*a*), where it was supposed that the rate constant for build up of activator varied instantaneously with membrane potential. Instead, we have assumed that the steady-state level of coupler approaches a new (voltage-dependent) equilibrium level, with a time constant which does not depend upon potential.

The depression of test calcium signals by hyperpolarizing conditioning pulses is less than predicted from a linear relationship between potential and steady-state coupler level. This may be taken into account by supposing that at potentials more negative than about −70 mV the relationship is non-linear, and that coupler level reaches some minimal value (possibly zero) below about −100 mV. From the discrepancy between the observed dependence of calcium release on conditioning potential and that predicted by the linear model, the steady-state voltage dependence of coupler level was derived over the range −50 to −140 mV (Fig. 11*B*). This varies from a slope of zero at potentials below about −100 mV, to linearity above about −60 mV. Unfortunately, it is not possible to explore potentials above −50 mV in this way, because of calcium release by the conditioning pulse. A linear relation is likely however, because of the good agreement between the linear model and experimental data for test depolarizations over this range.

The only unsatisfactory feature of the model is that we were forced to introduce an additional voltage-independent delay of about 1 msec into the coupling process in order to fit observations of the arsenazo signal latency. A part of this extra latency will be due to the fact that we estimated the latency from the point at which the tangent to the rising phase crossed the base line. The initial rise occurs earlier than this, but is difficult to determine accurately. Other factors which might be expected to contribute towards the latency include the T-tubule time constant, the reaction time of arsenazo III, and a possible reaction time of calcium gates in the s.r.

Time course of calcium release

We previously suggested that the time differential of the rising phase of the arsenazo signal may correspond to the time course of calcium release from the s.r. (Miledi *et al.* 1982). The coupler model allows the predicted time course of calcium release to be estimated, and we therefore compared this with experimental observations. Fig. 14 reproduces records from Fig. 8*B* of Miledi *et al.* (1982), showing the differentiated arsenazo signal elicited by an action potential. This gives a measure

of the rate of increase in free calcium, since the time constant of decline of the response (about 80 msec) is sufficiently slow that it may be neglected. The predicted time course of coupler build up and decay was calculated from eqn. 1 at intervals of 0·25 msec, and is shown in trace *c*. Calcium release was assumed to be linearly proportional to coupler level above a threshold equivalent to a steady-state potential of −50 mV (dashed line). This is indicated by the filled circles in *b*. The model trace has been normalized to the same peak height as the differentiated arsenazo recording, and is shifted rightwards by 1·2 msec. All parameters used in the calculation were derived from results in the present paper (time constants of coupler build up and decay = 3 and 5 msec, additional latency l = 1·2 msec).

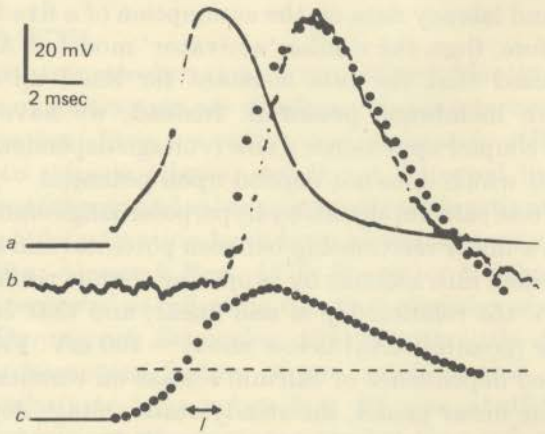


Fig. 14. Time course of release of calcium from the s.r. in response to an action potential. Traces show: *a*, membrane potential, *b*, experimental (noisy trace) and predicted (filled circles) time course of rate of release of calcium, *c*, predicted time course of build up and decay of coupler. Experimental records are taken from Fig. 8*B* of Miledi *et al.* (1982), and trace *b* is the time differential of the rising phase of the arsenazo response. Resting potential was −67 mV, and temperature 10 °C. Calculation of the predicted curves is explained in the text. Amplitude of traces in *b* is uncalibrated. The coupler level in *c* is scaled as the equivalent steady-state potential, and the dashed line indicates the threshold for calcium release at −50 mV.

The predicted time course of calcium release matches well with the observations, although several possible complicating factors were not taken into account. These include diffusion time of calcium ions, depletion of calcium in the s.r. and inactivation of the coupling process (Miledi *et al.* 1983). The additional latency of 1·2 msec is introduced somewhat arbitrarily, but is the same value as required to account for the latency following voltage–clamp pulses.

Relations with charge movement experiments

Recordings of non-linear charge movement from muscle fibres are thought to give an indication of an initial stage in e.–c. coupling which precedes calcium release (see Schneider, 1981; Adrian, 1978 for reviews). In terms of the coupler model, this charge movement might be expected to reflect the rates of build up and decay of coupler. Some simple comparisons are therefore worthwhile.

(i) The voltage dependence of the amount of charge moved (e.g. Fig. 9. Horowicz & Schneider, 1981*a*) is closely similar to the estimated voltage dependence of steady-state coupler level (Fig. 11*B*). Both show an approximately zero slope for potentials more negative than about −90 mV, increasing to a roughly linear relationship at potentials more positive than about −60 mV. In particular, both charge movement and coupler level show appreciable activation at potentials below threshold (about −50 mV) for detectable calcium release. At voltages near threshold for activation of charge movement or coupler build up, the potential changes required to give e-fold changes are 14 mV for coupler build up and between 8 mV (Chandler, Rakowski & Schneider, 1976*a*) and 17 mV (Horowicz & Schneider, 1981*b*) for charge moved.

(ii) Depolarizing pulses which give a threshold mechanical contraction produce equal charge movements, irrespective of pulse duration (Horowicz & Schneider, 1981*b*). These pulses would also be expected to give rise to a constant (threshold) level of coupler.

(iii) Sub-rheobase conditioning pre-pulses decrease the latency between onset of a test pulse and the time at which the total charge moved has exceeded the threshold corresponding to contractile activation (Horowicz & Schneider, 1981*b*). Conditioning pre-pulses similarly affect the calcium release latency (Fig. 7*B*; see also Kovacs *et al.* 1979).

(iv) The time constants for build up and decay of coupler are estimated as 3–5 msec (at 10 °C), and do not vary with potential over the range −110 to −60 mV. The time constants for decay of charge movements are in a similar range, for example about 10 msec for the 'on' measurement at −40 mV and 0–2 °C (Chandler *et al.* 1976*a*). However, the 'off' rates when returning to a hyperpolarized potential after depolarization are consistently faster than the 'on' rates, and both vary with potential.

(v) Adrian & Peres (1979) and Hui (1982) have dissected the 'on' charge movement into two parts, a main component (Q_β) which corresponds to the 'classical' charge movement, and a hump (Q_γ) which may reflect calcium release from the s.r. It is interesting that, while the voltage dependence of Q_β is similar to that for coupler build up, the voltage dependence of Q_γ resembles that for calcium release from the s.r.

Possible physical basis for the coupler model

The coupler model proposed here is similar in many respects to the 'activator' model of Adrian *et al.* (1969*a*), for which several possible mechanisms have been postulated (see Mathias, Levis & Eisenberg, 1980, for recent discussion). These fall into three principal categories: (i) The coupler represents the build up and decay of a substance, which acts as a messenger between tubular depolarization and calcium release from the s.r. Calcium ions crossing the tubular membrane have been postulated to be this messenger (Ford & Podolsky, 1972), although this now seems unlikely (Miledi *et al.* 1977*a*, *b*; Lüttgau & Spiecker, 1979). (ii) Charged molecules in the T-tubule membrane move under the influence of the electric field across the membrane, and cause release of calcium from the s.r. (Adrian, 1978; Schneider, 1981), possibly by means of a mechanical linkage (Chandler, Rakowski & Schneider 1976*b*). In this case the coupler would represent the distribution of charged particles in the T-tubule membrane. (iii) The T-tubules are electrically coupled to the s.r., and

depolarization of a fibre causes a potential change in the s.r. which leads to calcium release (Mathias *et al.* 1980). In this model, the coupler would represent the s.r. membrane potential.

There is little direct evidence at present for any of these mechanisms, but we favour either (ii) or (iii), simply because they are able to account for both the observed properties of calcium release and of non-linear charge movement. Fig. 15*A* presents a simple scheme, based on (iii) above, which is able to account for our data. This is entirely speculative, but provides an electrical analogue which may be helpful in thinking about models of e.–c. coupling, and has been chosen to be physiologically realistic. Calcium release is via a voltage-sensitive gate in the s.r. membrane, and is triggered by the potential V_{sr} across the membrane. Changes in potential across the T-tubule membrane V_T cause changes in V_{sr}, with a time constant given by $(1/g_c + 1/g_{sr})\, C_{sr}$, where g_c is the coupling conductance, g_{sr} the leakage resistance

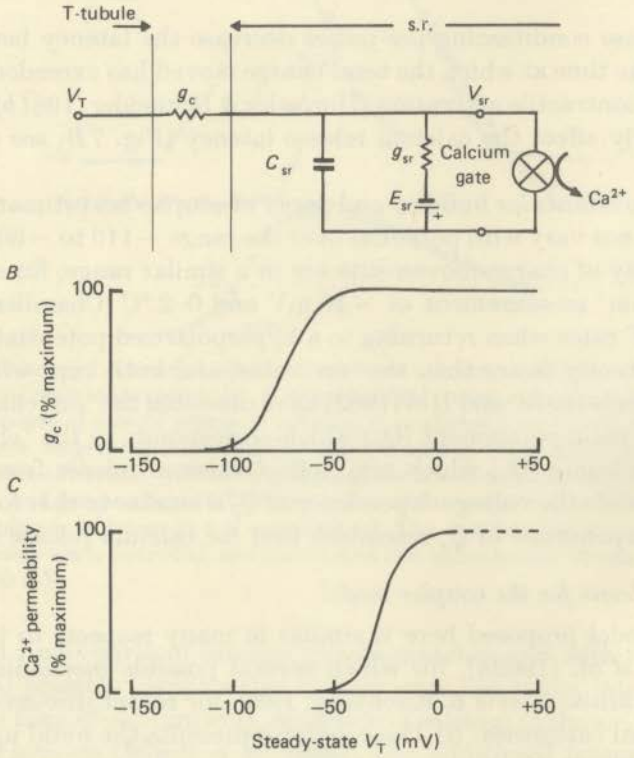


Fig. 15. Speculative model for e.–c. coupling. *A*, changes in potential across the T-tubule membrane (V_T) cause a current to flow through the coupling conductance (g_c) and hence alter the potential across the s.r. membrane (V_{sr}). C_{sr} represents the capacitance of the s.r. membrane, and g_{sr} is the leakage resistance across the membrane. Calcium release is via a voltage activated gate in the s.r. membrane. A resting potential (E_{sr}) is required across the s.r. membrane in this model, since g_c approaches zero at hyperpolarized membrane potentials, and without a biasing voltage, V_{sr} would then approach zero and trigger calcium release. *B*, voltage dependence of g_c. The curve was derived from the estimated relation between V_T and coupler level given in Fig. 11*B*. *C*, postulated dependence of calcium permeability of the calcium gate on V_T. The curve is the same as the sigmoid curve in Fig. 13*A*, showing the dependence of amount of calcium released on steady-state value of V_T.

across the membrane and C_{sr} the capacitance of the s.r. membrane. g_c is presumed to vary as a function of V_T, as shown in Fig. 15*B*. The dependence of calcium release upon V_T is attributed principally to the characteristics of the calcium gate, and is sketched in Fig. 15*C* for long depolarizing pulses, where V_{sr} would be linearly proportional to V_T. The model predicts that the time constant for coupler build up should vary with V_T, which is not in agreement with our results. However, if g_{sr} were greater than g_c during depolarization, then only small changes in time constant would be seen at different potentials. Most of our experiments were with small depolarizations, where g_c would be small.

The model in Fig. 15*A* is similar to the 'linear' model proposed by Mathias *et al.* (1980, 1981) to account for many of the characteristics of non-linear charge movement, and it is encouraging that observations with two very different techniques should lead to similar conclusions. Mathias *et al.* (1980) discuss several implications of this model, and it is not necessary to consider these further here.

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