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# EXTRACELLULAR IONS AND EXCITATION–CONTRACTION COUPLING IN FROG TWITCH MUSCLE FIBRES

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SUMMARY

1. Intracellular calcium transients were recorded from voltage-clamped frog twitch muscle fibres using Arsenazo III. The possible role of extracellular ions in excitation–contraction (e.–c.) coupling was examined using ion substitutions and blocking drugs in the bathing medium. Parameters measured included the Arsenazo response size to a standard depolarizing pulse (5 ms, 0 mV) and the strength–duration curve for threshold Arsenazo signal.

2. Addition of tetrodotoxin (TTX) decreased the response size to small (−30 mV, 5 ms), but not large (+30 mV, 10 ms) depolarizations, probably because of poor voltage clamp of the tubular membrane in the absence of TTX. Clamping TTX-treated fibres with the wave form of a recorded action potential gave an Arsenazo response similar to that elicited by the normal action potential (at 10 °C).

3. Complete substitution of sodium (by choline, lithium or Tris) or chloride (by methyl sulphate or maleate) in the bathing solution gave no appreciable changes in the size of the Arsenazo response.

4. Reduction of extracellular free $[Ca^{2+}]$ to low levels using EGTA caused a slight reduction in the calcium signal elicited by the standard depolarization (to 74 % after a few hours, and to 62 % after 2 days; temperature 5–10 °C). The strength–duration curve was unchanged.

5. Arsenazo responses about 75 % of the control size could be elicited in high potassium solution (42 mM-$K_2SO_4$) by strong (+80 mV, 20 ms) depolarizations, after re-polarizing the fibres to −90 mV for a few minutes. The voltage dependence of activation was shifted to more positive potentials in this solution.

6. Tetraethylammonium (TEA) bromide at a concentration of 20 mM did not alter the Arsenazo signal, whilst 120 mM-TEA reduced the response by 25 %. 3,4-diaminopyridine (DAP) reduced the size of the Arsenazo signal at a concentration of 5 mM, and caused spontaneous release of calcium from the sarcoplasmic reticulum (s.r.) in the absence of membrane potential changes. The Arsenazo signal elicited by an action potential was enhanced by 1 mM-DAP, because of prolongation of the action potential, but was depressed by higher concentrations.

7. We conclude that e.–c. coupling does not involve the influx of any external ions into the muscle fibre. If a current flow between the T-tubules and the s.r. is involved in e.–c. coupling, then this is probably carried by an efflux of potassium ions.

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## INTRODUCTION

A major problem in muscle physiology is the mechanism whereby depolarization of the T-tubules leads to release of calcium from the sarcoplasmic reticulum (s.r.) (for reviews see; Costantin, 1975; Endo, 1977; Caputo, 1978; Stephenson, 1981; Grinell & Brazier, 1981; Oetliker, 1982). Several schemes have been proposed, which may be divided into three types (Oetliker, 1982; Miledi, Parker & Zhu, 1983*b*): (i) excitation–contraction (e.–c.) coupling is achieved by the passage of a 'messenger' substance, which enters the fibre through the tubular membrane and activates the s.r. Entry of calcium ions (Sandow, 1952; Endo, Tanaka & Ogawa, 1970; Ford & Podolsky, 1972; Potreau & Raymond, 1980) or sodium ions (Caillé, Ildefonse & Rougier, 1978; Potreau & Raymond, 1982) has been suggested to be wholly or partially responsible for triggering e.–c. coupling; (ii) charged particles in the T-tubule membrane move under the influence of the electric field across the membrane, and this movement leads to the release of calcium from the s.r. (Schneider & Chandler, 1973; for reviews see Adrian, 1978; Schneider, 1981); (iii) the T-tubules are electrically coupled to the s.r., and depolarization causes a potential change in the s.r., which leads to calcium release (Mathias, Levis & Eisenberg, 1980).

Possible ways of discriminating between these hypotheses are provided by examining the effects of changes in ionic composition of the extracellular bathing fluid, and by using drugs to block specific transmembrane ion fluxes. Clearly, in scheme (i) above, if the entry of a messenger substance were prevented (either by its removal from the bathing fluid, or by blocking with a specific drug), then e.–c. coupling should be impaired or abolished. Scheme (iii) involves a current flow between T-tubules and s.r. (which must be carried by an ionic flux), and thus changes in extracellular ions might also be expected to have a considerable influence on e.–c. coupling. On the other hand, the charge movement in (ii) has no direct requirement for any ions other than to provide an electrically conducting path in the T-tubule lumen, although ions might modify the charge movement (e.g. by binding of divalent ions to the charged particles or screening of surface charges on the membrane).

We describe here an examination of the effects of ion substitutions and blocking drugs in the extracellular fluid on e.–c. coupling, using Arsenazo III as an intracellular indicator to monitor calcium release from the s.r. (Miledi, Parker & Schalow, 1977; Miledi, Parker & Zhu, 1982, 1983*a*, *b*). Our basic procedure was to voltage clamp fibres and measure the size of the intracellular calcium transient evoked by a fixed depolarizing pulse under various conditions. Additionally, strength–duration curves for the threshold Arsenazo signal were measured in some experiments, since we believe these give information about a time-dependent process in e.–c. coupling (Miledi *et al.* 1983*b*).

## METHODS

Experiments were made on the cutaneous pectoris muscle of *Rana temporaria*, at a temperature of 9–11 °C. Methods for using Arsenazo III to record intracellular calcium transients, and for voltage clamping fibres, were as described previously (Miledi *et al.* 1982, 1983*b*). Most experiments were performed by first obtaining control measurements from about six fibres with the muscle bathed in normal Ringer solution, and then examining several other fibres in a test bathing solution. The

TABLE 1. Composition of bathing solutions (mM)

| Ringer solution | NaCl | KCl | $Ca^{2+}$ | $MgCl_2$ | EGTA | HEPES | TEA | Other | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Normal | 120 | 2 | 2 (Cl) | — | — | 4 | Br | — | — | — |
| 0 calcium | 120 | 2 | — | 5 | 1 or 10 | 4 | Br | — | — | — |
| High calcium | 120 | 2 | 12 (Cl) | — | — | 4 | Br | — | — | — |
| High magnesium | 120 | 2 | 2 (Cl) | 5 | — | 4 | Br | — | — | — |
| Methyl sulphate | — | — | ($SO_4$) | — | — | 4 | $SO_4$ | Methyl Na sulphate 88 | Methyl K sulphate 2 | NaOH 75 |
| Choline | — | — | 2 (Cl) | — | — | 4 | Br | Choline chloride 120 | Tris base to set pH | — |
| Tris maleate | — | — | 4 (Cycl.) | — | — | — | Ac | Tris maleate 84 | Tris base 84 | — |
| TEA | — | 2 | 2 (Cl) | — | — | 4 | Br 120 | NaOH to set pH | — | — |
| Lithium | — | 2 | 2 (Cl) | — | — | 4 | — | LiCl 120 | Tris base to set pH | — |
| High potassium | — | 3 | 2 (Cycl.) | — | — | 4 | $SO_4$ | $K_2SO_4$ 42 | Sucrose 112 | KOH to set pH |

The TEA column indicates which TEA salt was used (bromide, sulphate or acetate); concentration was 20 mM except where indicated. In some experiments, as noted in the text, TEA was not added. No allowance was made for differences in osmolarity in the presence or absence of TEA. The $Ca^{2+}$ column indicates which calcium salt was used (chloride, sulphate or cyclamate (Cycl.)) and the total concentration of the salt. Calcium sulphate was a saturated solution. EGTA was added as the free acid. All solutions were titrated to pH 7·2, using NaOH except where otherwise indicated.

muscle was washed several times when changing solutions, and was allowed to equilibrate for at least 1 h in a new solution before taking readings. The compositions of the various solutions used are given in Table 1. Unless otherwise stated, all solutions included tetrodotoxin (TTX), $5 \times 10^{-7}$ g ml$^{-1}$ to block the sodium conductance, and hence improve clamp performance. For the same reason, tetraethylammonium (TEA) bromide (20 mM) was also added in most cases to reduce the delayed potassium current. In solutions containing low chloride concentrations, TEA acetate or sulphate was used instead of the bromide.

Amplitudes of Arsenazo optical records are expressed as the fractional absorbance change $\Delta A/A_{570}$, where $\Delta A$ is the calcium-dependent absorbance change at the wave-length pair 650–700 nm, and $A_{570}$ is the resting absorption of the injected dye at 570 nm. This expression gives a measure which is linearly proportional to the average free calcium concentration in the fibre, and standardizes for differences in fibre diameter and the amount of dye injected (Miledi *et al.* 1982). Under the conditions used, the Arsenazo signal reflects almost entirely free calcium changes, with negligible interference from magnesium, pH or dichroic changes (Miledi *et al.* 1982; Baylor, Chandler & Marshall, 1982*a*, *b*).

A standard depolarizing pulse of 5 ms duration to 0 mV from a holding potential of −75 mV was used to elicit Arsenazo responses. This was chosen to give an Arsenazo signal of similar size to that elicited by an action potential at a temperature of 10 °C. Mean response size to the pulse in normal Ringer solution was $\Delta A/A_{570} = 0{\cdot}107 \pm 0{\cdot}0063$ (± S.E. of the mean for twenty-three fibres), as compared to 0·095 for the action potential (Miledi *et al.* 1982). Unless otherwise stated, all results are given as mean ± 1 S.E. of mean with the number of fibres in parentheses.

Intervals of at least 60 s were allowed between stimuli, so that all responses were obtained from fibres in a rested-state condition (Miledi *et al.* 1983*a*). Strength–duration curves were obtained as described previously (Miledi *et al.* 1983*b*). Values for resting potentials were measured before penetration of the dye pipette.

## RESULTS

### *Sodium ions*

*Effect of TTX.* Most experiments were made with TTX ($5 \times 10^{-7}$ g ml$^{-1}$) present in the bathing fluid, as otherwise voltage-activated sodium currents made it difficult to maintain good voltage-clamp control. However, TTX could decrease contractions elicited by voltage-clamp depolarizations, possibly because of blocking of the tubular action potential (Bastian & Nakajima, 1974), or because of blocking of a sodium influx required to trigger e.–c. coupling (Caillé *et al.* 1978). We therefore examined Arsenazo signals from voltage-clamped fibres in the absence and presence of TTX.

Records were obtained from six fibres in normal Ringer solution without TTX, and then from a further seven fibres from the same muscle after addition of $5 \times 10^{-7}$ g TTX ml$^{-1}$. All solutions included 20 mM-TEA bromide (Fig. 1). A problem in interpreting the results is that, although the surface membrane potential was relatively well clamped in this experiment without TTX (Fig. 1*A*, *B*), deeper parts of the tubular system would be less well clamped, and may have depolarized more than the surface potential because of activation of the sodium conductance. To test this, we used three different depolarizing pulses. For the smallest (to −30 mV for 5 ms), activation of the tubular sodium conductance would tend to give a greater tubular depolarization than that recorded with the micro-electrode. On the other hand, the largest pulse (to +30 mV for 10 ms) should show little difference between tubular and surface potentials, since the depolarization was closer to the sodium equilibrium potential, and with a duration appreciably longer than that of the sodium conductance change.

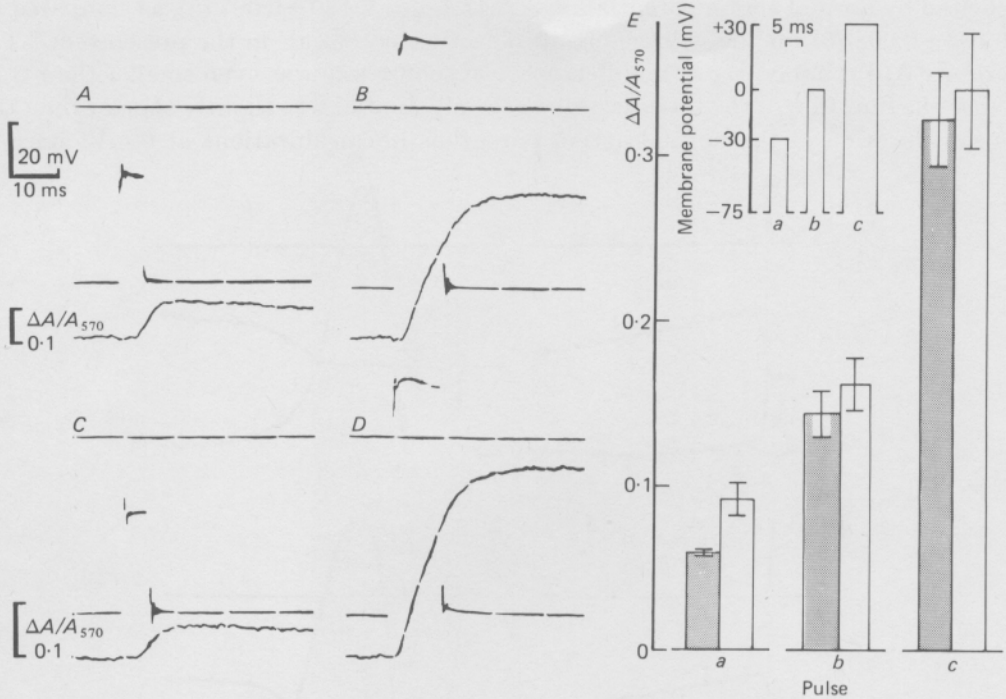


Fig. 1. Effect of TTX on size of Arsenazo responses elicited by different voltage-clamped depolarizing pulses. *A–D*, sample records showing responses to two different test pulses in the absence (*A–B*) and presence (*C–D*) of TTX ($5 \times 10^{-7}$ g ml$^{-1}$). Pulses correspond to the smaller and larger pulses shown in the inset in *E*. Calibration bars are: 20 mV, 10 ms and $\Delta A/A_{570} = 0{\cdot}1$. Note that the optical calibration is different in *A–B* and *C–D*, since the records were from two different fibres. *E*, mean response sizes elicited in normal Ringer solution with (shaded blocks) and without (open blocks) TTX. Three different pulses were used as shown in the inset (nominally, pulse $a = -30$ mV, 5 ms; $b = 0$ mV, 5 ms; $c = +30$ mV, 10 ms). Error bars give $\pm 1$ S.E. of the mean. Six fibres were examined without TTX, and seven with.

Fig. 1 *E* shows mean sizes of Arsenazo signals in the presence and absence of TTX. With the small pulse, addition of TTX caused a large reduction in response size (to 66 %), but raising the pulse potential to 0 mV diminished the effect of TTX (89 % of control), and with the largest pulse (+30 mV, 10 ms) the response in TTX was 95 %, and not significantly different from the control.

*Role of the tubular spike during twitches.* Bastian & Nakajima (1974) reported that the regenerative action potential in the tubules is responsible for about 70 % of the twitch tension at 20 °C, but plays little role at 10 °C. We were interested to repeat this experiment, using the intracellular calcium transient as a measure of activation.

Calcium transients were first recorded from five fibres bathed in normal Ringer solution, in response to directly elicited action potentials. The muscle was then treated with TTX ($5 \times 10^{-7}$ g ml$^{-1}$) and TEA (20 mM) and fibres were voltage clamped. An action potential previously recorded on FM tape was used as a command signal for the clamp. Typical records are shown in Fig. 2. Little difference in response size was seen between control and TTX-treated fibres. The mean Arsenazo response

elicited by normal action potentials was $\Delta A/A_{570} = 0{\cdot}129 \pm 0{\cdot}007$ (5), as compared to $0{\cdot}103 \pm 0{\cdot}008$ (5) for the voltage-clamped 'action potential' in the presence of TTX and TEA. Probably, the true difference in response size was even smaller than this, since the duration of the tape-recorded action potential was slightly shorter than the mean duration of the normal action potentials (mean durations at 0 mV; normal

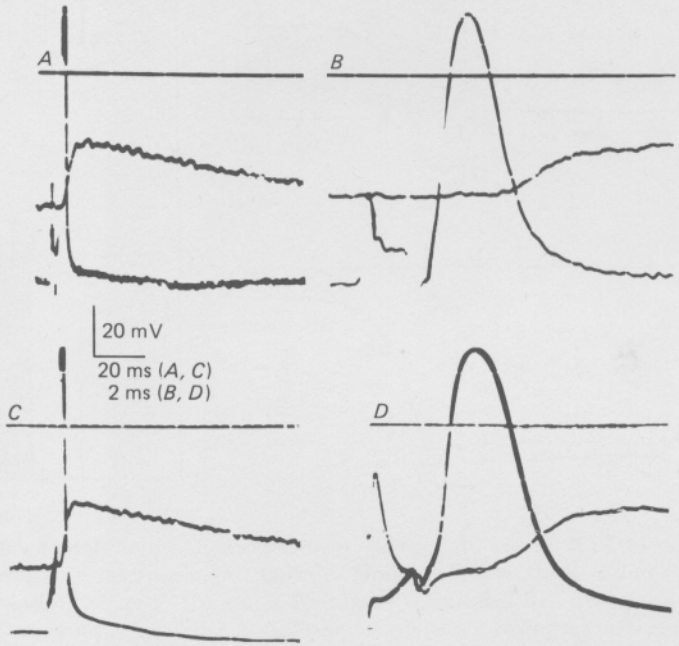


Fig. 2. Arsenazo signals evoked by normal action potentials ($C, D$), and voltage-clamped 'action potentials' in the presence of TTX and TEA ($A, B$). In each frame, the upper trace indicates the 0 mV reference line, the middle trace is Arsenazo light absorbance at 650–700 nm, and the lower trace is membrane potential. $A, B$, the fibre was stimulated under voltage clamp by the recorded wave form of an action potential. Bathing solution included TTX, $5 \times 10^{-7}$ g ml$^{-1}$ and 20 mM-TEA bromide. Both records from the same fibre, at different sweep speeds. Temperature 10 °C. $C, D$, the fibre was stimulated by a just-threshold depolarizing current pulse to give an action potential. Normal Ringer solution without TTX or TEA. The artifact at the beginning of the optical trace in $D$ is due to the reset circuit. Both records from the same fibre, at different sweep speeds.

action potential = $2{\cdot}1 \pm 0{\cdot}06$ ms, clamped 'action potential' = $1{\cdot}7 \pm 0{\cdot}04$ ms). Thus, the clamped 'action potential' duration was about 81 % of the control, whilst the clamped Arsenazo response size was 80 % of the control. The mean diameter of TTX-treated fibres was $48 \pm 4$ $\mu$m.

*Choline substitution.* Experiments were made on two muscles, using choline to substitute for sodium in the bathing solution. All solutions included TTX and TEA. Mean Arsenazo signals $\Delta A/A_{570}$ evoked by the standard depolarizing pulse (0 mV, 5 ms) were: muscle A, normal Ringer solution = $0{\cdot}095 \pm 0{\cdot}008$ (5), choline Ringer solution = $0{\cdot}098 \pm 0{\cdot}006$ (7); muscle B, normal Ringer solution = $0{\cdot}12 \pm 0{\cdot}03$ (3), choline Ringer solution = $0{\cdot}073 \pm 0{\cdot}011$ (8). Resting potentials of fibres were: control = $-79 \pm 1{\cdot}4$ mV, choline Ringer solution = $-98 \pm 2{\cdot}4$ mV. Muscle A there-

fore showed no apparent change in response size after changing to choline Ringer solution, and the decrease in muscle B is not significant at a 5% confidence level.

Strength–duration curves for the threshold Arsenazo signal were obtained from three fibres in muscle B in normal Ringer solution, and from five fibres after changing to choline Ringer solution (Fig. 3*A*). The rheobase potential was more negative in choline Ringer solution (control = −57 mV, choline = −65 mV), but otherwise there was little difference between the strength–duration curves. Strength–latency curves (the relationship between depolarizing potential and latency to onset of the Arsenazo signal: Miledi *et al.* 1983*b*) were also obtained from one fibre in control Ringer solution and two fibres in choline Ringer solution. No obvious differences were apparent.

*Tris maleate substitution.* Arsenazo signals were recorded from one muscle bathed in isotonic Tris maleate Ringer solution, where both sodium and chloride ions were substituted (20 mM-TEA acetate was present in this solution). Tris maleate was found to act as a calcium buffer, and a total of 4 mM-calcium was therefore added to this solution to give a free calcium concentration of about 1 mM (estimated by titration using Arsenazo III). Any errors in estimating this free calcium would not be important, since the size of the calcium transient in muscle is little affected by variation of the external calcium level over a very wide range (see later).

Resting potentials were: normal Ringer solution = −78·5 ± 2·0 mV (6); Tris maleate Ringer solution = −84·2 ± 4·8 mV (6). The mean size of the Arsenazo signal elicited by the standard depolarization ($\Delta A/A_{570}$) was 0·134 ± 0·008 (5) in normal Ringer solution, and 0·119 ± 0·009 (6) in Tris maleate solution. Strength–duration curves in Tris maleate Ringer solution were similar to the control (Fig. 3*B*), although the rheobase was slightly more negative (3 mV).

*Lithium substitution.* Replacement of sodium by lithium in Ringer solution has been reported to reduce the tension developed during a twitch, although with considerable variability (Caillé, Ildefonse, Rougier & Roy, 1981). We therefore measured Arsenazo signals from fibres bathed in lithium Ringer solution. The action potential is little changed in this solution (Caillé *et al.* 1981; and see later), so we used this as a stimulus, rather than the voltage-clamped pulse. TTX and TEA were not added to any solutions.

No significant change in size of the Arsenazo signal was seen. Mean responses ($\Delta A/A_{570}$) were: normal Ringer solution = 0·063 ± 0·005 (5); lithium Ringer solution = 0·068 ± 0·005 (7). Resting potentials were slightly lower in lithium Ringer solution (control = −85·8 ± 1·0 mV; lithium = −78 ± 0·5 mV), but only slight changes were seen in parameters of the action potential (overshoot = 30·7 mV in control and 24·1 mV in lithium, duration measured at 0 mV = 1·6 ms in control and 1·6 ms in lithium).

After returning the muscle to normal Ringer solution after bathing in lithium for 1·5 h, the Arsenazo signal was larger than the initial control value in normal Ringer solution ($\Delta A/A_{570}$ = 0·086 ± 0·008 (5)). This was probably a result of an increase in both action potential overshoot (34·8 mV) and duration (1·9 ms at 0 mV).

*Chloride ions*

Two experiments were made using different anions to replace chloride in the Ringer solution. The first used Tris maleate to replace both sodium and chloride, and is

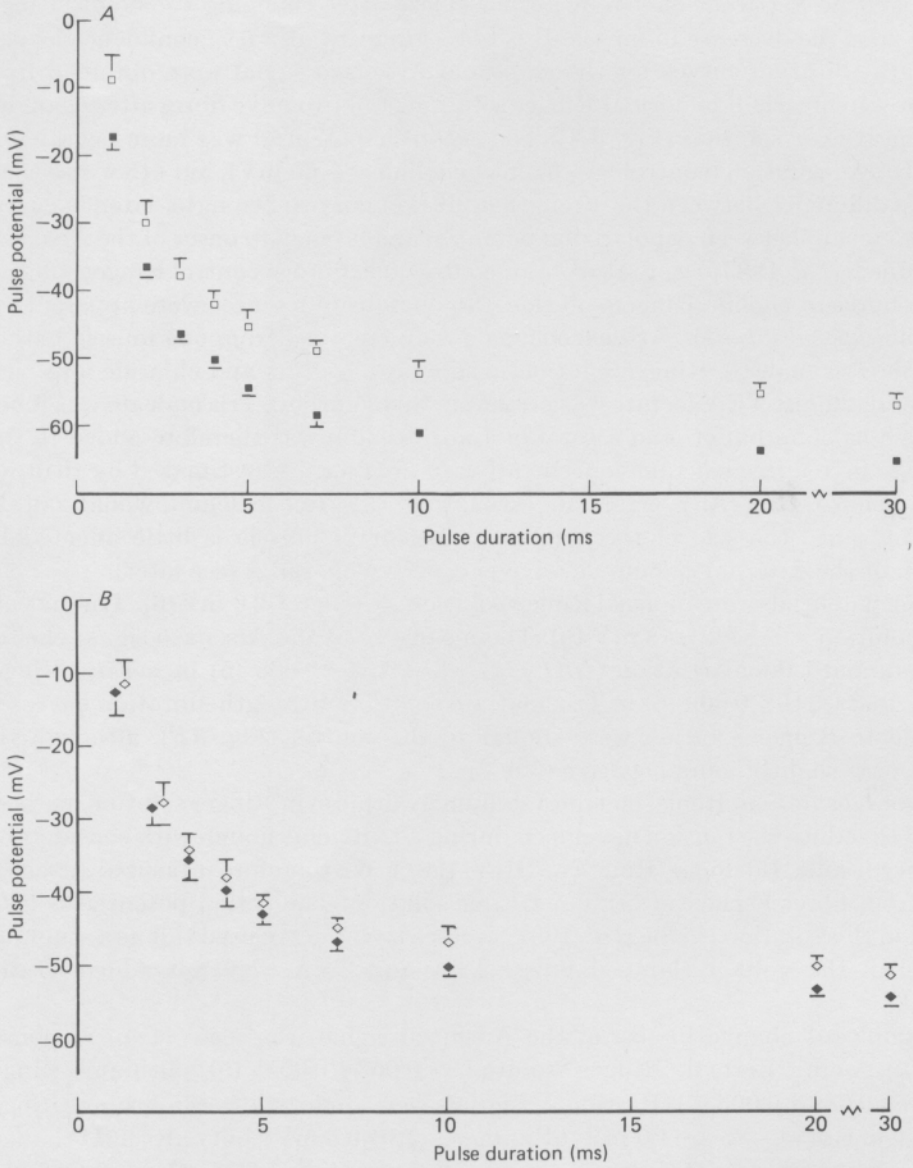


Fig. 3 *A* and *B*. For description see opposite.

described above. In the second, chloride ions alone were replaced by methyl sulphate. 20 mM-TEA sulphate was used in this solution, and saturated calcium sulphate was added to give a free calcium concentration of about 1 mM (Hodgkin & Horowicz, 1959, though recent data from Dani, Sanchez & Hille, 1983, indicates that the free calcium may have been as high as 4 mM).

Mean resting potential in the methyl sulphate solution was $-80{\cdot}2 \pm 1{\cdot}1$ mV (9), as compared to $-80{\cdot}3 \pm 1{\cdot}5$ mV (10) from fibres bathed in normal Ringer solution (two muscles). Arsenazo responses to the standard depolarization ($\Delta A/A_{570}$) were:

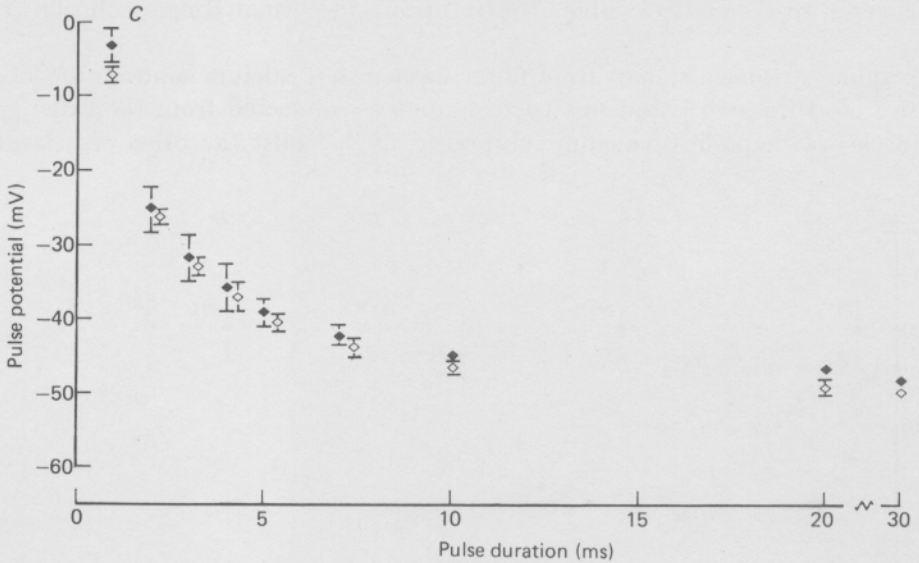


Fig. 3. Strength–duration curves showing the pulse potential required to elicit a just-detectable Arsenazo signal for any given pulse duration in various bathing solutions. In each case, open symbols show measurements from fibres bathed in normal Ringer solution, and filled symbols give measurements from other fibres in the same muscles, after changing to the ion-substituted solutions. Bars indicate 1 S.E. of mean; errors are smaller than the symbol width where not indicated. Pulse durations of 1, 2, 3, 4, 5, 7, 10, 20 and 30 ms were examined; some points have been displaced horizontally for clarity. *A*, data from three fibres in normal Ringer solution, and five fibres in choline solution. *B*, data from three fibres in normal Ringer solution, and three fibres in Tris maleate solution. *C*, data from three fibres in normal Ringer solution, and three fibres in methyl sulphate solution.

control = 0·082 ± 0·003 (10); methyl sulphate = 0·095 ± 0·008 (9). The difference was not statistically significant. Strength–duration curves were similar in control and methyl sulphate solutions (Fig. 3*C*).

*Calcium ions*

*Low calcium solutions.* Measurements were made of Arsenazo signals elicited by the standard depolarizing pulse (0 mV for 5 ms) from fibres which had been bathed for different times in a Ringer solution containing no added calcium and 1 mM-EGTA. Magnesium (5 mM) was added to this solution to maintain the resting potential and input resistance of the fibres. Addition of 5 mM-magnesium to normal Ringer solution caused no significant change in response size (Fig. 4*D*). Contaminating calcium in the 0 calcium Ringer solution was estimated as 8 $\mu$M before addition of EGTA (using Arsenazo III as an indicator, and assuming an apparent dissociation coefficient of 30 $\mu$M for the dye). The free calcium concentration after addition of EDTA should thus have been $< 10^{-8}$ M (Armstrong, Bezanilla & Horowicz, 1972).

Fibres bathed in 0 calcium solution for a few hours showed a reduction in the Arsenazo signal to about 74 % of the control value obtained from other fibres in the same muscle examined previously in normal Ringer solution (Fig. 4*A*). The response

size recovered to the control value after returning to normal Ringer solution (Fig. 4*A*).

To examine Arsenazo signals from fibres bathed in 0 calcium solution for longer times, we used the two cutaneous pectoris muscles dissected from the same frog. One muscle was kept in 0 calcium solution at 5 °C, whilst the other was treated

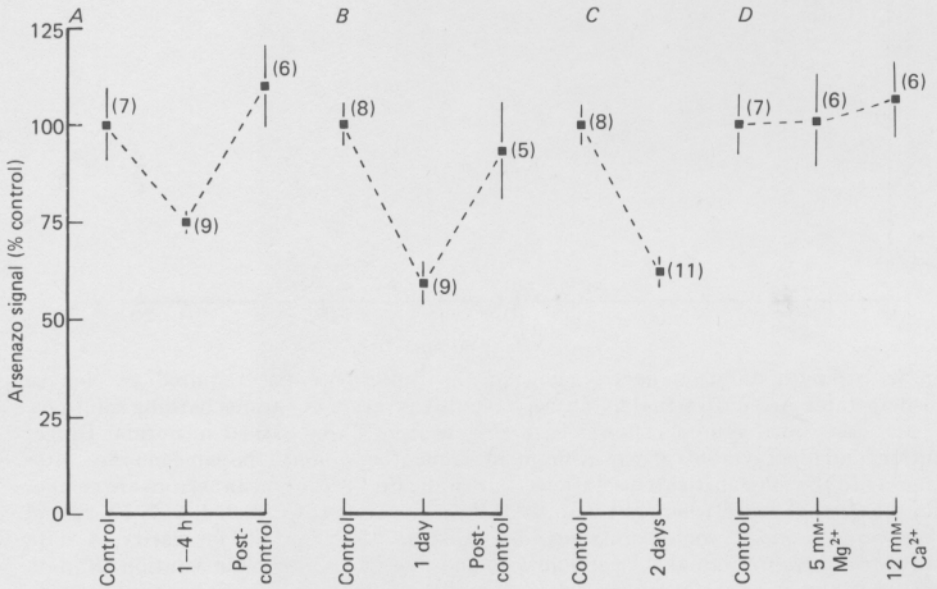


Fig. 4. Mean size of Arsenazo responses evoked by the standard test pulse (0 mV, 5 ms) in solutions with different divalent cation compositions. Results are expressed as a percentage of the control response size in normal Ringer solution. Bars are ±1 S.E. of the mean. Figures in parentheses give the number of fibres examined. *A*, fibres bathed in 0 calcium solution for 1–4 h, and then 1–2 h after returning to normal Ringer solution. *B*, the muscle was bathed in 0 calcium solution for about 24 h. Post-control measurements were obtained about 2 h after returning the muscle to normal Ringer solution. Control measurements were obtained from the paired muscle from the same frog, which had been kept in normal Ringer solution for 24 h. *C*, the same as in *B*, except that the muscle was maintained in 0 calcium Ringer solution for 2 days. *D*, response sizes after addition of 5 mM-magnesium, or an extra 10 mM-calcium, to normal Ringer solution.

identically apart from being bathed in normal Ringer solution, and was used to obtain control readings. After one day in 0 calcium Ringer solution the Arsenazo signal was reduced to 60% of the control, but recovered to close to the control value after returning to normal Ringer solution (Fig. 4*B*). Fibres bathed in 0 calcium solution for 2 days (separate experiment) showed no further reduction in response size (62% of control: Fig. 4*C*).

Resting potentials were little changed in the 0 calcium Ringer solution. The mean value after 2 days was $-86{\cdot}1 \pm 1{\cdot}4$ mV (10), compared to $-91{\cdot}5 \pm 0{\cdot}8$ mV (8) for fibres in the control muscle after 2 days.

Increasing the EGTA concentration in the 0 calcium Ringer solution to 10 mM gave no extra reduction in Arsenazo signal. This was examined in one experiment, where the action potential was used as the test stimulus, and responses were compared from

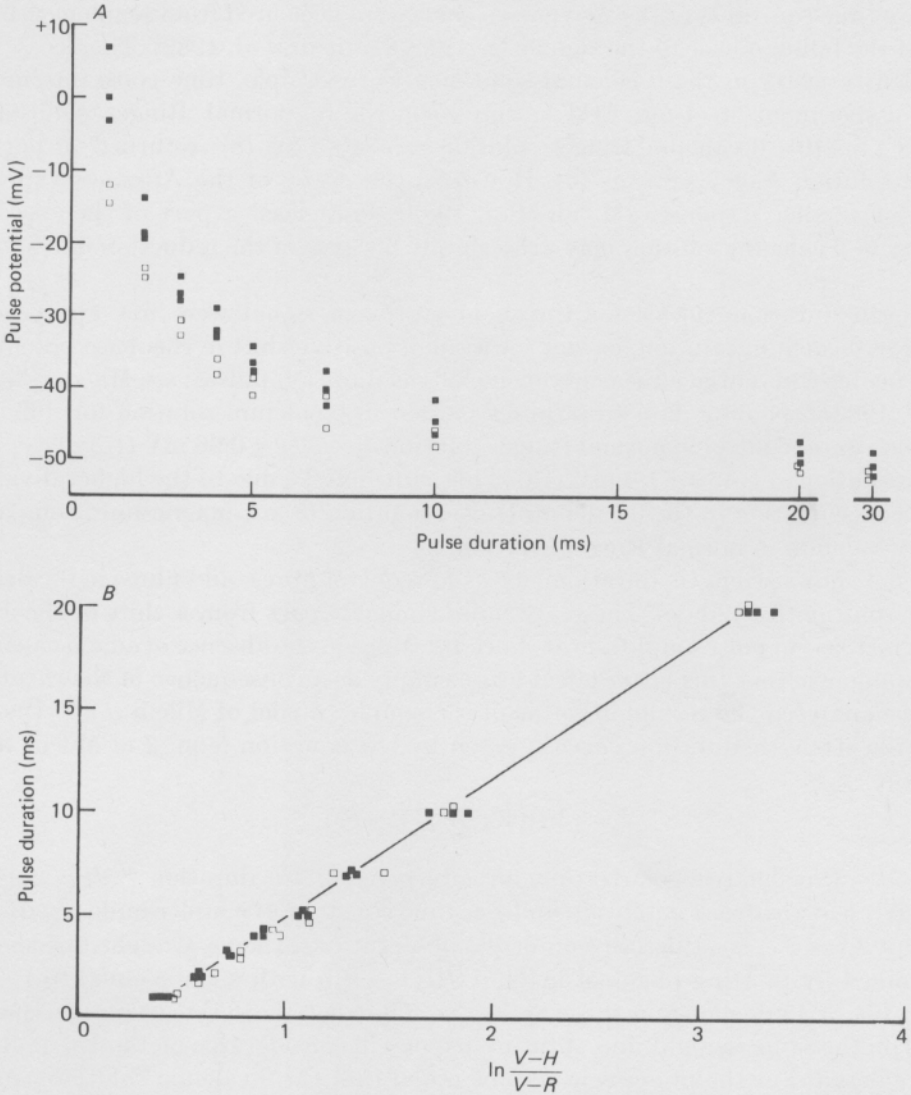


Fig. 5. Strength–duration curves for fibres bathed in 0 calcium plus 1 mM-EGTA Ringer solution for 24 h. *A*, data on linear coordinates. Filled symbols are measurements from three fibres in 0 calcium Ringer solution, while open symbols are from the paired muscles which had been in normal Ringer solution for 24 h. *B*, the same data as in *A*, but re-plotted with the horizontal axis giving $\ln((V-H)/(V-R))$. See text for further explanation. Some points are slightly displaced vertically for clarity. The rheobase potential ($R$) was measured for each fibre, and the holding potential ($H$) was $-75$ mV for all fibres. The line was drawn by eye, and corresponds to a time constant of 6·8 ms for an e-fold change in $(V-H)/(V-R)$.

paired muscles soaked for 1 day in 0 calcium Ringer solution containing either 1 mM- or 10 mM-EGTA. Mean response sizes were; 1 mM-EGTA = $0{\cdot}033 \pm 0{\cdot}0047$ (6); 10 mM-EGTA = $0{\cdot}034 \pm 0{\cdot}006$ (5).

Raising the calcium concentration in normal Ringer solution to 12 mM caused a very slight increase in Arsenazo signal (Fig. 4*D*), which is not statistically significant.

Decay time constants of the Arsenazo signals were measured from semilogarithmic plots of the falling phase of the signals (see Fig. 6, Miledi *et al.* 1982). The decay was consistently faster in the 0 calcium solutions. For example, time constants in the 1 day experiment in 1 mM-EGTA were: control in normal Ringer solution = $66{\cdot}3 \pm 8{\cdot}4$ ms (6); 0 calcium Ringer solution = $50{\cdot}4 \pm 4$ ms (8); returned to normal Ringer solution = $68{\cdot}7 \pm 8{\cdot}4$ ms (5). However, the decay of the Arsenazo signal is faster for smaller responses (Miledi *et al.* 1982), so at least a part of the speeding of decay in 0 calcium solution may arise simply because of the reduction in response size.

Strength–duration curves for threshold Arsenazo signal were not appreciably altered in 0 calcium solution, except for a small positive shift in rheobase potential. Mean rheobase potentials (measured using 30 ms duration pulses; see Miledi, Parker & Zhu, 1983*c*), pooling data from fibres bathed in 0 calcium solution for different durations were: control in normal Ringer solution = $-50{\cdot}2 \pm 0{\cdot}96$ mV (15); 0 calcium Ringer solution = $-45{\cdot}4 \pm 1{\cdot}0$ mV (13). This shift may be due to the higher divalent cation concentration in the 0 calcium Ringer solution (5 mM-magnesium, compared to 2 mM-calcium in normal Ringer solution.

Fig. 5*A* shows strength–duration curves for control fibres, and fibres in 0 calcium Ringer solution for 2 days. These are quite similar, apart from a shift in rheobase and an increase in pulse amplitude at short durations in the absence of added calcium. To examine whether this latter effect arose simply as a consequence of the rheobase shift, we analysed the results in terms of the coupler model of Miledi *et al.* (1983*b*), where the strength–duration curve is given by the equation (eqn. 2 of Miledi *et al.* (1983*b*)):

$$t = \tau \ln ((V-H)/(V-R)), \tag{1}$$

where $V$ = threshold depolarization for any given pulse duration $t$, $H$ = holding potential, $R$ = rheobase potential, and $\tau$ = time constant of coupler build-up. If this equation gives a correct description of the observations, then a straight line should be obtained by plotting $t$ against $\ln ((V-H)/(V-R))$, with a slope equal to $\tau$. The data of Fig. 5*A* are shown in this way in Fig. 5*B*, where both sets of measurements lie well on the same straight line. A future paper will consider this method of analysis in more detail. For the present, it may be noted that the 0 calcium solution caused almost no change in the strength–duration relationship apart from the shift in rheobase, and that the time constant of coupler build-up estimated from the slope of the line was 6·8 ms in both control and 0 calcium solutions.

Similar measurements of strength–duration curves were made from fibres bathed in 0 calcium Ringer solution for a few hours and for 1 day, and these also showed no significant differences from control fibres (apart from rheobase). The over-all mean value of the time constant for coupler build-up in fibres in 0 calcium Ringer solution was $5{\cdot}75 \pm 0{\cdot}3$ ms (12), compared to $6{\cdot}4 \pm 0{\cdot}7$ ms (10) for control fibres.

*Effect of D600.*The organic 'calcium antagonist' D600 (methoxyverapamil) has been shown to block calcium currents in muscle (Sanchez & Stefani, 1978; Almers & Palade, 1981), although its action is not particularly specific (Bregestovski, Miledi & Parker, 1980). The size of the Arsenazo signal elicited by the standard depolarization (5 ms, 0 mV, from $-75$ mV; temperature 10 °C) was only slightly reduced by

concentrations of D600 considerably in excess of that sufficient to block the slow calcium current in muscle (30 $\mu$M; Almers & Palade, 1981). Mean peak values of $\Delta A/A_{570}$ at different concentrations of D600 were: control = 0·125 ± 0·006 (13); 50 $\mu$M-D600 = 0·12 ± 0·015 (5); 200 $\mu$M-D600 = 0·107 ± 0·008 (10) and 400 $\mu$M-D600 = 0·104 ± 0·006 (5). Only one measurement was obtained from each fibre.

*Potassium ions*

*High external potassium.* The potassium concentration in normal Ringer solution is low (2 mM), so that any potassium flux involved in e.–c. coupling will be in an outward direction, and removal of external potassium would not be expected to have much effect on e.–c. coupling. In agreement with this, there was little change in the size of the calcium transient elicited by the standard depolarization in fibres bathed in potassium-free Ringer solution for about 1 h ($\Delta A/A_{570}$ = 0·12 ± 0·004 (7) in normal Ringer solution, and 0·12 (2) in 0 potassium Ringer solution). The rheobase potential was also unchanged (normal Ringer solution = −50·8 ± 0·7 mV (6); 0 potassium Ringer solution = −49·7 ± 0·5 mV (3)). Instead, we therefore examined the effects of increasing the potassium concentration in the bathing fluid, so that any potassium efflux should be reversed or reduced.

The bathing solution used contained principally $K_2SO_4$ and sucrose (Table 1). This was selected to have: (i) high (81 mM) potassium concentration; (ii) the same $[K^+] \times [Cl^-]$ product as normal Ringer solution; (iii) isotonicity with normal Ringer solution and (iv) the same ionic strength as normal Ringer solution. TTX and TEA acetate were added to both Ringer and high potassium solutions at the usual concentrations. Muscles were slackened to rest length when changing to the high potassium solution, to avoid damage due to contracture.

No adverse effects on the calcium transient were apparent following treatment in the high potassium solution for a few hours. This was tested in one experiment by recording Arsenazo signals to a fixed depolarizing pulse (0 mV for 20 ms from a holding potential of −110 mV) whilst bathing fibres initially in normal Ringer solution, and then after returning to normal Ringer solution following immersion in high potassium solution for 2·5 h. Mean response sizes ($\Delta A/A_{570}$) were: control = 0·304 ± 0·043 (7); normal Ringer solution after high potassium = 0·306 ± 0·03 (6).

The mean resting potential of fibres bathed in the high potassium solution was −15·6 ± 0·3 mV (8). At this potential, e.–c. coupling is inactivated (Caputo, 1978), and in order to record Arsenazo signals we voltage-clamped fibres at −90 mV for at least 5 min before taking readings. This time would have been sufficient for almost complete repriming of the e.–c. coupling process. The time course of repriming of the Arsenazo signal to test depolarizations (0 mV for 10 or 20 ms) was examined in four fibres, which showed an approximately exponential recovery with a mean time constant of about 20 s.

Fibres were very easily damaged in the high potassium solution, and often broke down during dye injection or after stimulation. We therefore concentrated on obtaining Arsenazo responses to two fixed stimuli (0 mV and +80 mV for 20 ms from a holding potential of −90 mV). Control measurements using the same pulse parameters were obtained in normal Ringer solution. Mean response sizes ($\Delta A/A_{570}$)

from three muscles were: normal Ringer solution, 0 mV = 0·341 ± 0·025 (15), +80 mV = 0·413 ± 0·03 (13); high potassium, 0 mV = 0·117 ± 0·014 (15), +80 mV = 0·272 ± 0·017 (13). Thus, the response size to the 0 mV pulse was considerably reduced in the high potassium solution (to 34 %), but with the +80 mV pulse the reduction was less (to 66 %).

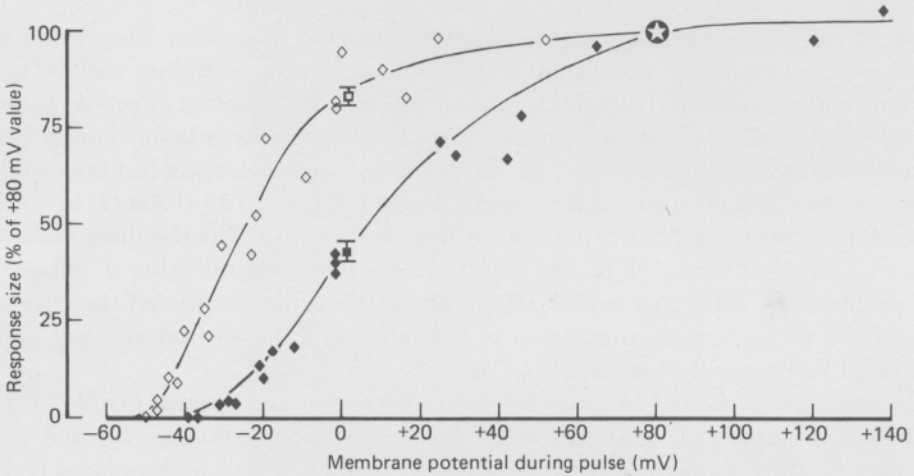


Fig. 6. Relationship between size of Arsenazo signal and membrane potential during a 20 ms duration depolarizing pulse, for fibres bathed in normal Ringer solution (open symbols) and high potassium solution (filled symbols). Data are expressed as a percentage of the mean response size for the +80 mV pulse in each solution. Points marked (◇) were obtained from three fibres bathed in normal Ringer solution, and points marked (◆) were obtained from three other fibres in the same muscle after changing to high potassium solution. Data points with error bars at 0 mV are mean values (±1 S.E. of the mean) from fifteen fibres in normal Ringer solution and thirteen fibres in high potassium solution (three muscles). Both solutions included TTX ($5 \times 10^{-7}$ g $ml^{-1}$) and TEA (20 mM). Holding potential was −90 mV. Temperature 10 °C. Curves drawn by eye.

The voltage dependence of the Arsenazo signal in normal Ringer solution and high potassium solutions is shown in Fig. 6, expressed as a percentage of the +80 mV value in each solution. Because of the fragility of the fibres in high potassium, the full curve was obtained from only three fibres, but mean values at 0 mV are included in Fig. 6 from thirteen fibres. In the high potassium solution, the strength–response curve is shifted to more positive potentials, and is less steep than in normal Ringer solution. The threshold potential is only about 10 mV more positive, whilst the potential for half-maximal activation is about 30 mV more positive.

*Tetraethylammonium and 3,4-diaminopyridine.* An alternative way to examine the role of any potassium current in e.–c. coupling is to use drugs known to block potassium channels. We used tetraethylammonium bromide (TEA) and 3,4-diaminopyridine (DAP), which both block the delayed rectifier potassium channel in muscle (Stanfield, 1970; Stefani & Chiarandini, 1982). It was important also to know whether 20 mM-TEA affected the Arsenazo signal, since this was routinely used to improve clamp performance.

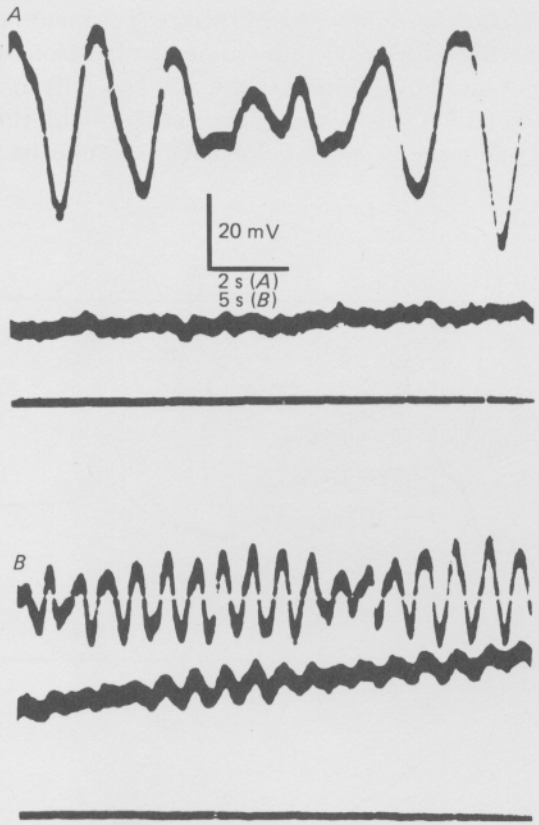


Fig. 7. Spontaneous contractions and oscillations of intracellular calcium from a fibre in the presence of 10 mM-DAP. In both frames the traces from top to bottom are: (i) light transmission at 700 nm, which gives an indication of movement during contractions, (ii) light transmission at 650–700 nm, which gives a calcium-dependent signal from Arsenazo loaded-fibres, and (iii) membrane potential. *A*, record obtained before injection of Arsenazo. Movements during spontaneous contractions caused fluctuations in the 700 nm trace, but these are almost completely cancelled out in the 650–700 nm trace. *B*, record from the same fibre after loading with Arsenazo III. The 650–700 nm trace now shows fluctuations, reflecting changes in intracellular calcium. No changes in membrane potential are seen in either record. Calibrations: 20 mV for both records, sweep speed 2 s in *A* and 5 s in *B*; fractional change in light transmission $\Delta I = 0{\cdot}054$ for both records. The upward drift in the 650–700 nm traces is due to the electronics, and does not necessarily imply a slow rise in intracellular calcium.

The effect of 20 mM-TEA bromide was examined in two muscles, with TTX ($5 \times 10^{-7}$ g ml$^{-1}$) present in all solutions. The mean Arsenazo response to the standard depolarization in control fibres (normal Ringer solution) was $\Delta A/A_{570} = 0{\cdot}109 \pm 0{\cdot}008$ (9), and after addition of TEA $= 0{\cdot}111 \pm 0{\cdot}006$ (7). No changes were apparent in the strength–duration curve after addition of TEA.

However, 20 mM-TEA blocks only about 80 % of the delayed rectifier potassium current (Stanfield, 1970), so we examined also the effect of bathing muscles in 120 mM-TEA bromide. The rheobase potential in this solution was shifted to more negative values, with a mean of about $-67$ mV (control $= -55$ mV). A holding

potential of −90 mV was therefore used in these experiments (for both control and TEA solutions), since there appeared to be some inactivation of e.–c. coupling when the normal holding potential of −75 mV was used. The shift in rheobase presumably arises from an action of TEA ions, since replacement of chloride by bromide causes only a slight shift (3 mV more negative) in rheobase for mechanical activation (Kao

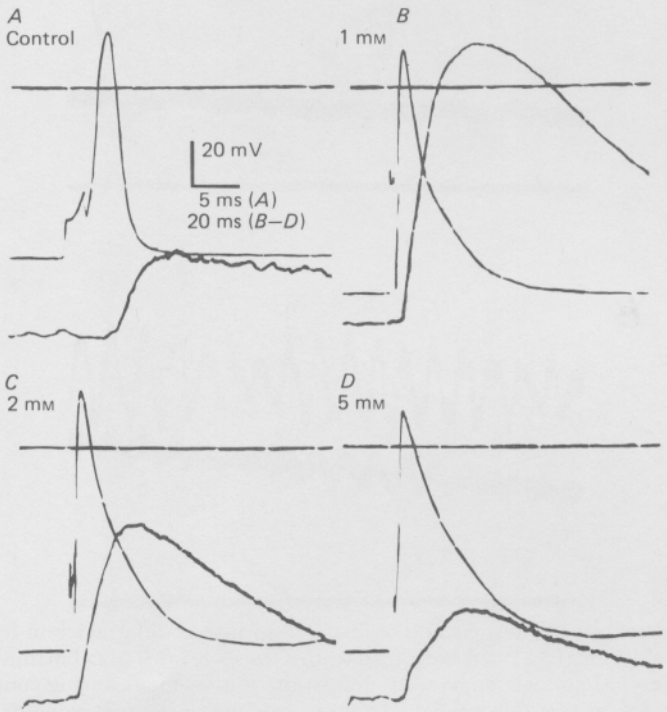


Fig. 8. Arsenazo signals evoked by action potentials in the presence of different concentrations of DAP. *A–D*, records showing action potentials (middle traces) and Arsenazo optical signals (lower traces). The upper lines in each frame indicate 0 mV potential. Record *A* was obtained in normal Ringer solution without DAP. The other records were obtained from other fibres in the same muscle at concentrations of DAP of: $B = 1$ mM, $C = 2$ mM, $D = 5$ mM. Fibres were stimulated by passing just supra-threshold depolarizing current pulses through the dye pipette. Note that the time scale in *A* is faster than for the other records (5 ms and 20 ms, respectively). Peak response sizes ($\Delta A/A_{570}$) were: $A = 0{\cdot}084$, $B = 0{\cdot}29$, $C = 0{\cdot}22$ and $D = 0{\cdot}12$.

& Stanfield, 1968). Mean Arsenazo responses to the standard depolarization (5 ms to 0 mV from −90 mV) were: normal Ringer solution $\Delta A/A_{570} = 0{\cdot}097 \pm 0{\cdot}008$ (5), and 120 mM-TEA $= 0{\cdot}073 \pm 0{\cdot}004$ (6).

Aminopyridines are effective at lower concentrations than TEA at blocking potassium currents in muscle (Stefani & Chiarandini, 1982). Arsenazo signals elicited by the standard depolarization (0 mV, 5 ms from −75 mV) were recorded at various concentrations of DAP in fibres from one muscle. Values of $\Delta A/A_{570}$ were: control $= 0{\cdot}112 \pm 0{\cdot}01$ (4); 0·5 mM-DAP $= 0{\cdot}106 \pm 0{\cdot}007$ (5); 2 mM-DAP $= 0{\cdot}091 \pm 0{\cdot}007$ (7); 5 mM-DAP $= 0{\cdot}035 \pm 0{\cdot}002$ (3); returned to normal Ringer solution

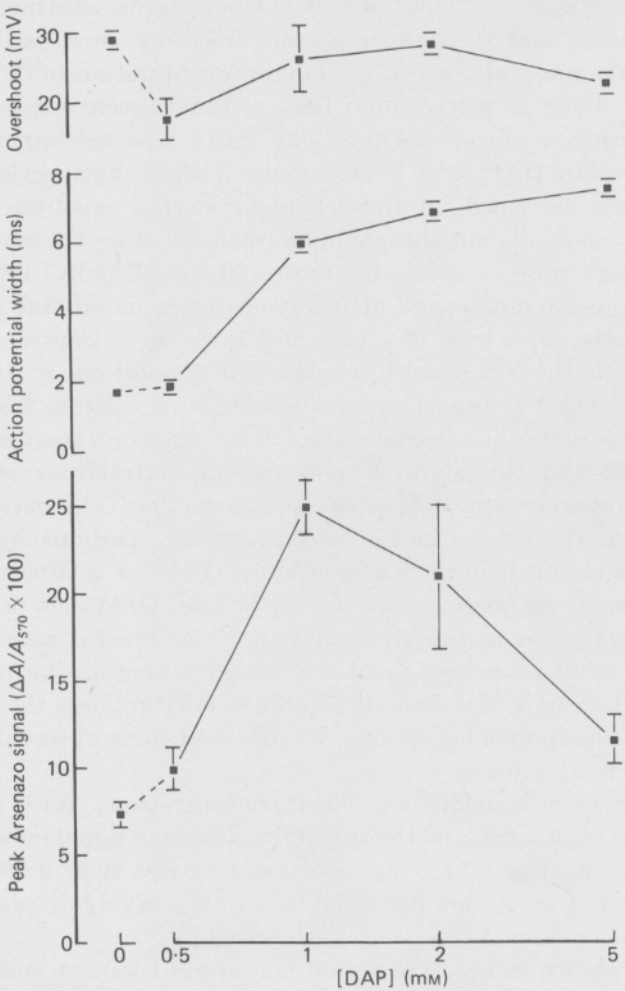


Fig. 9. Changes in mean action potential parameters and size of Arsenazo signal with different concentrations of DAP. Concentrations are plotted on a logarithmic scale. Plots show, from top to bottom, action potential overshoot, action potential duration (measured at 0 mV), and peak size of Arsenazo signal. Each point is a mean from five to six fibres, and bars indicate $\pm 1$ S.E. of the mean. Errors are smaller than the symbol width where not shown.

after DAP = $0{\cdot}098 \pm 0{\cdot}008$ (5). Thus, concentrations of DAP up to 2 mM caused little change in Arsenazo response, but 5 mM-DAP produced a large depression, which was almost completely reversible upon returning to normal Ringer solution. The reduction in size in 5 mM-DAP was confirmed in two other muscles, and the mean size was 19% of the control (seventeen fibres; three muscles).

A complication was that fibres often developed spontaneous contractions a few minutes after raising the DAP concentration to 5 mM. This spontaneous activity did not seem to be responsible for the decrease in Arsenazo signal, since the measurements given above were obtained from fibres showing no visible spontaneous contractions.

At a concentration of 10 mM-DAP, waves of spontaneous contractions were seen in virtually all fibres, and these were accompanied by slow oscillations in the intracellular free calcium level (Fig. 7). The maximum amplitude of these fluctuations corresponded to a change in intracellular free calcium concentration about 13% of that occurring during a normal twitch. The mean free calcium level was also presumably increased by DAP, since fluctuations fell below the mean level of the trace (Fig. 7*B*). One possible cause of these contractions is repetitive firing in the presynaptic nerve terminals, but this could be ruled out since the contractions were not abolished by high concentrations of curare ($10^{-5}$ g $ml^{-1}$) or TTX ($10^{-5}$ g $ml^{-1}$). Also, the contractions did not depend upon any change in membrane potential, since this showed no fluctuations, even at a gain of 0·5 mV $cm^{-1}$. Contractions were still present in muscles bathed in 0 calcium plus EGTA solution, so the most likely explanation is that DAP causes spontaneous release of calcium from the s.r. We examined one muscle which had been de-tubulated by glycerol treatment (Eisenberg & Eisenberg, 1968) and this showed spontaneous contractions when the DAP concentration was raised to 20 mM. It seems, therefore, that DAP may enter the fibre and directly act on the s.r. to cause calcium release, perhaps by blocking the potassium-selective channels in the s.r. membrane (Labarca & Miller, 1981).

*Effect of DAP on the calcium transient during twitches.* DAP has been reported to augment the twitch tension in directly stimulated chick muscle, an effect which was presumed to arise from prolongation of the action potential due to block of the delayed rectifier (Harvey & Marshall, 1977). Since DAP reduces the calcium signal elicited by voltage-clamp depolarizations, we repeated these observations using the action potential as a stimulus.

Fig. 8*A–D* shows records at different DAP concentrations. With 1 mM-DAP the action potential was prolonged, and the size of the Arsenazo signal increased by about three times. Increasing the DAP concentration to 2 mM then 5 mM caused little further prolongation of the action potential, but progressively decreased the size of the Arsenazo signal.

Mean values are shown in Fig. 9 of action potential overshoot and duration, and size of the Arsenazo signal, at different DAP concentrations. DAP appears to have two opposing effects on the intracellular calcium transient evoked by an action potential. At low concentrations (< 1 mM) the transient is augmented, due to prolongation of the action potential (without any change in overshoot), whilst at higher concentrations the inhibition of e.–c. coupling by DAP becomes apparent.

## DISCUSSION

This paper is concerned with the effects of extracellular ions on e.–c. coupling, and the results are discussed below under the headings of the various ions. Our basic premise was that if a transmembrane flux of any ion forms an essential stage in e.–c. coupling, then the blocking of this flux (by ion substitution or by blocking drugs) should abolish or very substantially reduce the intracellular calcium transient. Substitution of some ions gave small changes in size of the calcium transient and changes in rheobase potential. We have not investigated these effects in detail, but think that they result from a modulation of e.–c. coupling, rather than indicating an essential role for the ions in the coupling process.

Most measurements were made using depolarizations giving Arsenazo signals similar to those evoked during a single twitch, and we have not yet considered any possible role of extracellular ions during prolonged depolarization or tetanic stimulation.

*Sodium ions*

Our results do not provide evidence for supposing that extracellular sodium ions play any role in the e.–c. coupling process between tubular depolarization and calcium release from the s.r. Complete substitution of sodium in the Ringer solution by choline, lithium or Tris did not appreciably reduce the size of the Arsenazo signal evoked by standard depolarizations (voltage-clamped pulses to 0 mV for 5 ms, or the action potential in the case of lithium). Furthermore, blocking of the voltage-activated sodium conductance by TTX did not appear to affect the calcium transient, except for changes which could be attributed to the abolition of the regenerative depolarization in the tubules. An additional argument against the involvement of a sodium influx is given by the observation that depolarizations more positive than the sodium equilibrium potential do not cause any reduction in size of the intracellular calcium transient (Fig. 6: see also Miledi *et al.* 1977, 1983*b*; Eusebi, Miledi & Takahashi, 1983).

A possible role for sodium ions in e.–c. coupling has previously been suggested by two groups: (i) Potreau & Raymond (1982) found evidence for a sodium-induced mechanism of calcium release from the s.r. However, the sodium current they measured would probably be too slow to play any role in e.–c. coupling during twitches. Also, this current could only be recorded in a 0 calcium plus EGTA solution, and is of doubtful importance under normal conditions. A possible mechanism for this slow sodium current is suggested by recent observations that in very low external free calcium concentration, sodium ions may pass through channels which are normally highly selective for calcium (Almers, McClesky & Palade, 1982); (ii) Caillé *et al.* (1978) recorded a tubular sodium current using a double sucrose-gap clamp, which they proposed was partly responsible for tension development during a twitch. Our results do not support this idea, and it is possible that their findings may have arisen from poor voltage-clamp control of the tubular potential. Under our experimental conditions we found that the calcium transient evoked by small depolarizations (−30 mV) was considerably reduced by addition of TTX, probably because the tubules were poorly clamped in the absence of TTX and were being further depolarized by activation of the tubular sodium conductance. However, by using large depolarizations (+30 mV), where errors in clamping of the tubules are expected to be less serious, we found little reduction in the Arsenazo signal after adding TTX. Caillé *et al.* (1981) also report that substitution of sodium by lithium considerably reduces twitch tension. Using the Arsenazo signal as a measure of activation we found no change, and at present we do not understand this discrepancy. One possibility is that the entry of lithium ions into the cytoplasm might directly affect the contractile machinery.

An interesting finding is that the regenerative action potential in the tubules appears to play almost no role in e.–c. coupling in frog twitch fibres at 10 °C (fibre diameter 50 $\mu$m). The size of the calcium transient was closely similar when evoked by the recorded wave form of an 'action potential' in voltage-clamped fibres treated with TTX and TEA, or when elicited by a normal action potential. This is in

agreement with Bastian & Nakajima (1974), who used tension development as a measure of activation.

*Chloride ions*

Replacement of chloride ions in the Ringer solution by methyl sulphate or maleate caused no appreciable decrease in Arsenazo signal to the standard depolarization, and it is therefore unlikely that chloride plays any direct role in e.–c. coupling.

*Calcium ions*

Changes in external free calcium concentration over a very wide range had only a slight effect on the Arsenazo signal evoked by the standard depolarization. For example, reducing the free calcium concentration in the Ringer solution from 10 mM to about $10^{-8}$ M (in the presence of 1 mM-EGTA and 5 mM-magnesium) gave a reduction in peak response size by only about 30 %. This finding is in agreement with several previous observations that low external free calcium has little effect on twitch tension (Armstrong *et al.* 1972; Sandow, Krishna, Pagala & Sphicer, 1975; Lüttgau & Spieker, 1979), or on the intracellular calcium transient (Miledi *et al.* 1977; Blinks, Rudel & Taylor, 1978; Miledi, Nakajima, Parker & Takahashi, 1981; Eusebi *et al.* 1983). Some reports have suggested that low external free calcium may disrupt e.–c. coupling (Barrett & Barrett, 1978; Frank, 1982), but in these cases magnesium was not added to the bathing solutions, and the disruption is likely to have been due to damage to the muscle membrane in the absence of any extracellular divalent cations (Lüttgau & Spieker, 1979).

Calcium influx into a muscle fibre through ionic channels is expected to vary linearly with external free calcium concentration (Meves, 1968; Miledi, Parker & Schalow, 1980). It therefore seems most unlikely that a calcium influx could trigger e.–c. coupling, since the Arsenazo signal was only slightly reduced when the extracellular free calcium concentration was lowered by a factor of about one million. The small reduction in signal instead most probably arose from some secondary effect of extracellular divalent cations on the e.–c. coupling process.

Additional strong evidence against the involvement of a calcium influx is given by the fact that depolarizations to high positive potentials fail to reduce the size of the intracellular calcium transient (Fig. 6: see also Miledi *et al.* 1977, 1983*b*; Eusebi *et al.* 1983), even though the electrochemical driving force for calcium entry would have been much reduced or suppressed (Katz & Miledi, 1966). These results form a striking contrast with the mechanism at presynaptic nerve terminals, where an influx of calcium ions triggers the release of transmitter. Here, the transmitter release varies steeply with extracellular calcium concentration, is reduced by the presence of magnesium, and is suppressed by depolarization close to the calcium equilibrium potential (Katz, 1966; Katz & Miledi, 1966).

Miyamoto & Racker (1982) propose that the lack of effect of low calcium solutions on e.–c. coupling may not invalidate the 'trigger calcium' hypothesis, because even in the presence of EGTA, the free calcium concentration in the T-tubule lumen might be maintained as a result of active pumping of calcium across the tubular membrane together with a restricted access between the tubular lumen and the external solution. This argument would still not account for the fact that strong depolarizations do not

reduce the calcium transient. Also, we were able to record Arsenazo signals from fibres which had been bathed in 1 mM-EGTA for 2 days, while a rough calculation indicates that the amount of calcium pumping required to maintain the free calcium level in the tubules would deplete all the muscle calcium within a few hours.

In the presence of 1 mM-EGTA, a total calcium concentration of more than 1 mM would be required in the tubular lumen to give a free calcium concentration sufficient to allow the 'trigger calcium' mechanism to function. Since the tubular volume comprises about 0·4 % of the total fibre volume (Peachey, 1965) and the total calcium concentration in muscle fibres is about 1 mM (Endo, 1977), this means that at least 0·4 % of the muscle calcium would initially have to be pumped into the tubules to saturate the EGTA. A half-exchange time of about 5 s has been estimated for calcium movement between the tubules and the bathing solution (Fig. 12, Almers, Fink & Palade, 1981). Assuming that exchange of calcium–EGTA and EGTA occurs at a roughly similar rate, a further 0·2 % of the muscle calcium would have to be pumped into the tubules every 5 s in order to maintain the free calcium concentration. Thus, all of the calcium in the muscle fibres would be lost within about 100 min.

Our finding that the 'calcium antagonist' D600 has little effect on the Arsenazo signal elicited by a single brief depolarization is in agreement with some previous studies of calcium blocking drugs on contractile activation (Lüttgau & Spiecker, 1979; Gonzalez-Serratos, Valle-Aguilera, Lathrop & del Carmen Garcia, 1982), although after repeated stimulation, D600 may block contraction (Eisenberg, McCarthy & Milton, 1983). However, since the drug has several non-specific effects on muscle, including the blocking of sodium and potassium currents and the generation of spontaneous contractions (Bregestovski *et al.* 1980), a blocking of contraction by D600 does not necessarily indicate the involvement of a calcium channel in e.–c. coupling.

*Potassium ions*

The voltage dependence of activation of calcium release from the s.r. is shifted to more positive potentials in high potassium solution (Fig. 6), whilst the response elicited by strong depolarizations is only slightly reduced. This effect would be expected if an efflux of potassium were involved in e.–c. coupling, but it is difficult to exclude the possibility that these changes in voltage dependence of activation arose from some secondary consequence of the prolonged immersion of fibres in high potassium solution.

A shift of roughly 30 mV for half-maximal activation was found in 42 mM-$K_2SO_4$ solution as compared to normal Ringer solution. A shift of this magnitude is not expected from any surface charge effects due to replacement of sodium and chloride by potassium and sulphate (Kao & Stanfield, 1968; Hille, Woodhull & Shapiro, 1975). Also, any change in surface charge due to a high free calcium concentration in this solution (possibly 4 mM; see earlier) would be less than 5 mV (Hille *et al.* 1975). A large positive shift is, however, expected if a potassium efflux from the fibre is involved in e.–c. coupling. The potassium concentration in the s.r. is about 140 mM (Somlyo, Schuman & Somlyo, 1977). Thus, any potassium flux from either the myoplasm or the s.r. to the tubular lumen would always be outwards during activation of e.–c. coupling in normal Ringer solution, whilst in the 42 mM-potassium sulphate solution the flux would be expected to be outwards only at potentials more positive than about −15 mV. For example, at a potential of 0 mV, activation of e.–c.

coupling by a putative potassium current would be reduced in high external potassium, because of the lower concentration gradient. A problem with this interpretation, however, is that we could elicit Arsenazo signals at a potential of −30 mV, where an inward potassium current would be expected.

The finding that the voltage dependence of e.–c. coupling is altered in high potassium solutions may present a complication in interpretation of experiments using potassium contractures (see also Miledi *et al.* 1983*c*).

TEA gave no reduction in calcium transient size at a concentration of 20 mM, even though the delayed rectifier potassium current would have been reduced by about 80% (Stanfield, 1970). This is in agreement with previous work indicating that e.–c. coupling does not depend upon the delayed rectifier (Stanfield, 1970). There is little reason to suppose, however, that a putative potassium channel between the T-tubule and s.r. would be blocked by TEA in a similar manner to the delayed rectifier. DAP reduces the Arsenazo signal at low concentrations (5 mM), thus lending some support to the notion of an involvement of a potassium current in e.–c. coupling.

*Models of e.–c. coupling*

We find no evidence to indicate that an *influx* of any ion from the bathing solution into a muscle fibre plays a role in e.–c. coupling, since removal or substitution of each of the ions present in normal Ringer solution caused little change in the intracellular calcium transient. Models involving the entry of a 'messenger' substance which triggers calcium release from the s.r. (scheme (i) in the Introduction) therefore seem unlikely.

As regards models where a current flow from the T-tubule lumen into the s.r. activates calcium release (scheme (iii) in the Introduction), our results suggest that if such a current exists, it must be carried predominantly by an efflux of potassium ions. As discussed above, experiments with high potassium and DAP give some support to this model, but the arguments are not strong. At present, we feel the evidence is not sufficient to discriminate between models of e.–c. coupling involving current flow (scheme (iii)) or charge movement (scheme (ii)).

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REFERENCES

ADRIAN, R. H. (1978). Charge movement in the membrane of striated muscle. *Ann. Rev. Biophys. Bioeng.* **7**, 85–112.

ALMERS, W., FINK, R. & PALADE, P. T. (1981). Calcium depletion in frog muscle tubules: The decline of calcium current under maintained depolarization. *J. Physiol.* **312**, 177–207.

ALMERS, W., MCCLESKEY, E. W. & PALADE, P. T. (1982). Frog muscle membrane: a cation permeable channel blocked by micromolar external [$Ca^{2+}$]. *J. Physiol.* **332**, 52–53*P*.

ALMERS, W. & PALADE, P. T. (1981). Slow calcium and potassium currents across frog muscle membrane: measurements with a vaseline-gap technique. *J. Physiol.* **312**, 159–176.

ARMSTRONG, C. M., BEZANILLA, F. M. & HOROWICZ, P. (1972). Twitches in the presence of ethylene glycol bis (b-amino ether)-N,N′-tetraacetic acid. *Biochim. biophys. Acta* **267**, 605–608.

BARRETT, J. N. & BARRETT, E. F. (1978). Excitation–contraction coupling in skeletal muscle: blockade by high extracellular concentrations of calcium buffers. *Science, N.Y.* **200**, 1270–1272.

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. (1982*a*). Use of metallochromic dyes to measure changes in myoplasmic calcium during activity in frog skeletal muscle fibres. *J. Physiol.* **331**, 139–177.

BAYLOR, S. M., CHANDLER, W. K. & MARSHALL, M. W. (1983*b*). Dichroic components of Arsenazo III and Dichlorophosphonazo III signals in skeletal muscle fibres. *J. Physiol.* **331**, 179–210.

BLINKS, J. R., RUDEL, R. & TAYLOR, S. R. (1978). Calcium transients in isolated amphibian skeletal muscle fibres. Detection with aequorin. *J. Physiol.* **277**, 291–323.

BREGESTOVSKI, P. D., MILEDI, R. & PARKER, I. (1980). Blocking of frog endplate channels by the organic calcium antagonist D600. *Proc. R. Soc.* B **211**, 15–24.

CAILLÉ, J., ILDEFONSE, M. & ROUGIER, O. (1978). Existence of a sodium current in the tubular membrane of frog twitch muscle fibre; Its possible role in the activation of contraction. *Pflügers Arch.* **374**, 167–177.

CAILLÉ, J., ILDEFONSE, M., ROUGIER, O. & ROY, G. (1981). Surface and tubular membrane currents in frog twitch muscle fibres: implication in excitation contraction coupling. In *Adv. Physiol. Sci.*, vol. 5, ed. VERGARA, E., KOVÁCS, T. & KOVÁCS, L., pp. 389–409. Budapest: Pergamon Press.

CAPUTO, C. (1978). Excitation and contraction processes in muscle. *Ann. Rev. Biophys. Bioeng.* **7**, 63–83.

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

DANI, J. A., SANCHEZ, J. A. & HILLE, B. (1983). Lyotropic anions. Na channel gating and Ca electrode response. *J. gen. Physiol.* **81**, 255–281.

EISENBERG, B. & EISENBERG, R. S. (1968). Selective disruption of the sarcotubular system in frog sartorius muscle. *J. Cell Biol.* **39**, 451–467.

EISENBERG, R. S., MCCARTHY, R. T. & MILTON, R. L. (1983). Paralysis of frog skeletal muscle fibres by the calcium antagonist D600. *J. Physiol.* **341**, 495–505.

ENDO, M. (1977). Calcium release from the sarcoplasmic reticulum. *Physiol. Rev.* **57**, 77–108.

ENDO, M., TANAKA, M. & OGAWA, Y. (1970). Calcium induced release of calcium from the sarcoplasmic reticulum of skinned skeletal muscle fibres. *Nature, Lond.* **228**, 34–36.

EUSEBI, F., MILEDI, R. & TAKAHASHI, T. (1983). Aequorin–calcium transients in frog twitch muscle fibres. *J. Physiol.* **340**, 91–106.

FORD, L. E. & PODOLSKY, R. J. (1972). Intracellular calcium movements in skinned muscle fibres. *J. Physiol.* **223**, 21–33.

FRANK, G. B. (1982). The effects of reducing the extracellular calcium concentration on the twitch in isolated frogs skeletal muscle fibres. *Jap. J. Physiol.* **32**, 589–608.

GONZALEZ-SERRATOS, H., VALLE-AGUILERA, R., LATHROP, D. A. & DEL CARMEN GARCIA, M. (1982). Slow inward calcium currents have no obvious role in muscle excitation–contraction coupling. *Nature, Lond.* **298**, 292–294.

GRINNELL, A. D. & BRAZIER, M. A. (1981). *The regulation of muscle contraction: excitation–contraction coupling.* New York: Academic Press.

HARVEY, A. L. & MARSHALL, I. G. (1977). The facilitatory actions of aminopyridines and tetraethylammonium on neuromuscular transmission and muscle contractility in avian muscle. *Naunyn-Shmiedebergs Arch. Pharmacol.* **299**, 53–60.

HILLE, B., WOODHULL, A. M. & SHAPIRO, B. I. (1975). Negative surface charges near sodium channels of nerve: divalent ions, monovalent ions and pH. *Phil. Trans. R. Soc. B* **270**, 301–318.

HODGKIN, A. L. & HOROWICZ, P. (1959). The influence of potassium and chloride ions on the membrane potential of single muscle fibres. *J. Physiol.* **148**, 127–160.

KAO, C. Y. & STANFIELD, P. R. (1968). Actions of some anions on electrical properties and mechanical threshold of frog twitch muscle. *J. Physiol.* **198**, 291–309.

KATZ, B. (1966). *Nerve muscle and synapse.* New York: McGraw Hill.

KATZ, B. & MILEDI, R. (1966). A study of synaptic transmission in the absence of nerve impulses. *J. Physiol.* **192**, 407–436.

LABARCA, P. & MILLER, C. (1981). A $K^+$-selective, three-state channel from fragmented sarcoplasmic reticulum of frog leg muscle. *J. Membrane Biol.* 31–38.

LÜTTGAU, H. CH. & SPIEKER, W. (1979). The effects of calcium deprivation upon mechanical and electrophysiological parameters in skeletal muscle fibres of the frog. *J. Physiol.* **296**, 411–429.

MATHIAS, R. T., LEVIS, R. A. & EISENBERG, R. S. (1980). Electrical models of excitation–contraction coupling and charge movement in skeletal muscle. *J. gen. Physiol.* **76**, 1–31.

MEVES, H. (1968). The ionic requirements for production of action potentials in *Helix pomatia* neurones. *Pflügers Arch.* **304**, 215–241.

MILEDI, R., NAKAJIMA, S., PARKER, I. & TAKAHASHI, T. (1981). Effects of membrane polarization on sarcoplasmic calcium release in skeletal muscle. *Proc. R. Soc.* B **213**, 1–13.

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

MILEDI, R., PARKER, I. & SCHALOW, G. (1980). Transmitter induced calcium entry across the post-synaptic membrane at frog end-plates measured using Arsenazo III. *J. Physiol.* **300**, 197–212.

MILEDI, R., PARKER, I. & ZHU, P. H. (1982). Calcium transients evoked by action potentials in frog muscle fibres. *J. Physiol.* **333**, 655–679.

MILEDI, R., PARKER, I. & ZHU, P. H. (1983*a*). Calcium transients in frog skeletal muscle fibres following conditioning stimuli. *J. Physiol.* **339**, 223–242.

MILEDI, R., PARKER, I. & ZHU, P. H. (1983*b*). Calcium transients studied under voltage-clamp control in frog twitch muscle fibres. *J. Physiol.* **340**, 649–680.

MILEDI, R., PARKER, I. & ZHU, P. H. (1983*c*). Changes in threshold for calcium transients in frog skeletal muscle fibres due to calcium depletion in the T-tubules. *J. Physiol.* **344**, 233–241.

MIYAMOTO, H. & RACKER, E. (1982). Mechanism of calcium release from skeletal sarcoplasmic reticulum. *J. Membrane Biol.* **66**, 193–201.

OETLIKER, H. (1982). An appraisal of the evidence for a sarcoplasmic reticulum membrane potential and its relation to calcium release in skeletal muscle. *J. Muscle Res. & Cell Motility*, **3**, 247–272.

PEACHEY, L. D. (1965). The sarcoplasmic reticulum and transverse tubules of the frog's satorius. *J. Cell Biol.* **25**, 209–231.

POTREAU, D. & RAYMOND, G. (1980). Calcium-dependent electrical activity and contraction of voltage-clamped frog single muscle fibres. *J. Physiol.* **307**, 9–22.

POTREAU, D. & RAYMOND, G. (1982). Existence of a sodium-induced calcium release mechanism on frog skeletal muscle fibres. *J. Physiol.* **333**, 463–480.

SANCHEZ, J. A. & STEFANI, E. (1978). Inward calcium current in twitch muscle fibres of the frog. *J. Physiol.* **283**, 197–209.

SANDOW, A. (1952). Excitation–contraction coupling in muscular response. *Yale J. Biol. Med.* **25**, 176–201.

SANDOW, A., KRISHNA, M., PAGALA, D. & SPHICER, E. C. (1975). Excitation–contraction coupling: Effects of 'zero' calcium medium. *Biochem. biophys. Acta* **404**, 157–163.

SCHNEIDER, M. F. (1981). Membrane charge movement and depolarization-contraction coupling. *Ann. Rev. Biophys. Bioeng.* **7**, 85–112.

SCHNEIDER, M. F. & CHANDLER, W. K. (1973). Voltage dependent charge movement in skeletal muscle: A possible role in excitation–contraction coupling. *Nature, Lond.* **242**, 244–246.

SOMLYO, A. V., SCHUMAN, H. & SOMLYO, A. P. (1977). Composition of sarcoplasmic reticulum in situ by electron probe X-ray microanalysis. *Nature, Lond.* **268**, 556–558.

STANFIELD, P. R. (1970). The effect of the tetraethylammonium ion on the delayed currents of frog skeletal muscle. *J. Physiol.* **209**, 209–229.

STEFANI, E. & CHIARANDINI, D. J. (1982). Ionic channels in skeletal muscle. *Ann. Rev. Physiol.* **44**, 357–372.

STEPHENSON, E. W. (1981). Activation of fast skeletal muscle: contribution of studies on skinned fibres. *Am. J. Physiol.* **240**, C1–19.