Proc. R. Soc. Lond. B **229**, 39–46 (1986)
Printed in Great Britain

Minimal latency of calcium release in frog twitch muscle fibres

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(*Received* 21 *April* 1986)

Intracellular release of calcium in frog skeletal muscle fibres was monitored by the use of arsenazo III, in response to voltage clamped depolarizing pulses. A latency of a few milliseconds was evident between the onset of depolarization and the first detectable rise in the arsenazo–calcium signal, and this decreased logarithmically as the depolarization was increased. The minimal latency with strong depolarization (to +20 to +100 mV) was about 2 ms at 5 °C. This delay appears to be sufficiently long to be compatible with a chemically mediated coupling mechanism between depolarization and calcium release from the sarcoplasmic reticulum.

INTRODUCTION

When a muscle fibre is depolarized, by an action potential or a voltage clamped pulse, a clear latency is seen between the onset of depolarization and the first detectable rise in intracellular calcium as monitored by metallochromic dyes (Miledi *et al.* 1977, 1979, 1982, 1983*b*; Kovács *et al.* 1979; Palade & Vergara 1982; Baylor *et al.* 1982. This latency varies with temperature (Miledi *et al.* 1979), degree of depolarization (Kovács *et al.* 1979; Miledi *et al.* 1983*b*), and tonicity of the bathing solution (Parker & Zhu 1986). We had previously reported that the latency reduced with increasing amplitude of depolarizing pulses, reaching a minimum of about 2 ms at high positive potentials (at 10 °C and in normal Ringer) (Miledi *et al.* 1983*b*). However, those measurements were made by estimating the times at which a tangent to the main rising phase of the calcium–arsenazo III signal intercepted the baseline, and this would have tended to overestimate the true latency, because of the initially curved foot of the response. To overcome this problem we have repeated those measurements, with improved resolution of the calcium–arsenazo III optical signal and using signal averaging, so as to allow the latency to the first detectable signal to be more accurately determined. Estimation of the minimum latency between depolarization and the onset of calcium release from the sarcoplasmic reticulum has recently become more important, because of

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the suggestion that the link between these two events may be chemically mediated (Vergara *et al.* 1985; Volpe *et al.* 1985), and would thus presumably involve some irreducible delay (cf. Katz & Miledi 1965).

Methods

Experiments were performed on the cutaneous pectoris muscle of *Rana temporaria*. Details of the preparation, and of the methods of voltage clamping and recording intracellular calcium transients by the use of arsenazo III were as described previously (Miledi *et al.* 1982, 1983*a*,*b*). 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 voltage clamp control. For these experiments, fibres were injected with large amounts of arsenazo III (the absorbance of injected dye at 570 nm was 0.1–0.2), to increase the size of the calcium signals. Such intracellular concentrations of dye have an appreciable buffering effect on intracellular calcium (Miledi *et al.* 1982), but this was immaterial for the present purpose of measuring the initial rise of the calcium signal. Muscle fibres were usually clamped at a potential of −75 mV between stimuli, to avoid complications from nonlinear behaviour of the e.–c. coupling system at more negative potentials (Miledi *et al.* 1983*b*). The temperature of the Ringer solution was controlled by Peltier elements in the base of the chamber, and was monitored by a thermocouple (Comark Ltd) placed close to the edge of the muscle. Records of arsenazo signal latencies were filtered at 2 kHz by a simple *RC* circuit, and averaged by using a Data Labs DL 400B signal averager. Illustrations in figures were traced by hand from photographs of the display screen.

Results

Latency of the calcium transient

Figure 1 shows the rising phase of averaged arsenazo signals elicited by voltage clamp depolarization to 0 mV (at 10 °C). The response to a 3 ms pulse showed an initial curvature preceding the main, roughly linear, rising phase (figure 1*a*). As a result of this the earliest detectable rise in signal (figure 1*b*) occurred about 1 ms before the onset that would be estimated by taking the tangent to the main rising phase (figure 1*a*). Despite the initial curvature, the onset of the arsenazo signal was quite abrupt when examined at high gain (figure 1*b*). This indicates the existence of a definite latency, rather than of a gradual rise beginning at the onset of the pulse, which might give the appearance of a latency by being initially masked by the noise level. Certainly, a first order (exponentially rising) process could not account for records such as those in figures 1 and 2.

Two other interesting features are evident in figure 1. The first is that for short pulse durations (a few milliseconds) such as those illustrated, the major part of the rise in arsenazo signal occurred after the end of the pulses. Indeed, with the 1.3 ms pulse the signal did not begin until the pulse had ended. This behaviour was seen consistently in many fibres, although we were never able to find a convincing

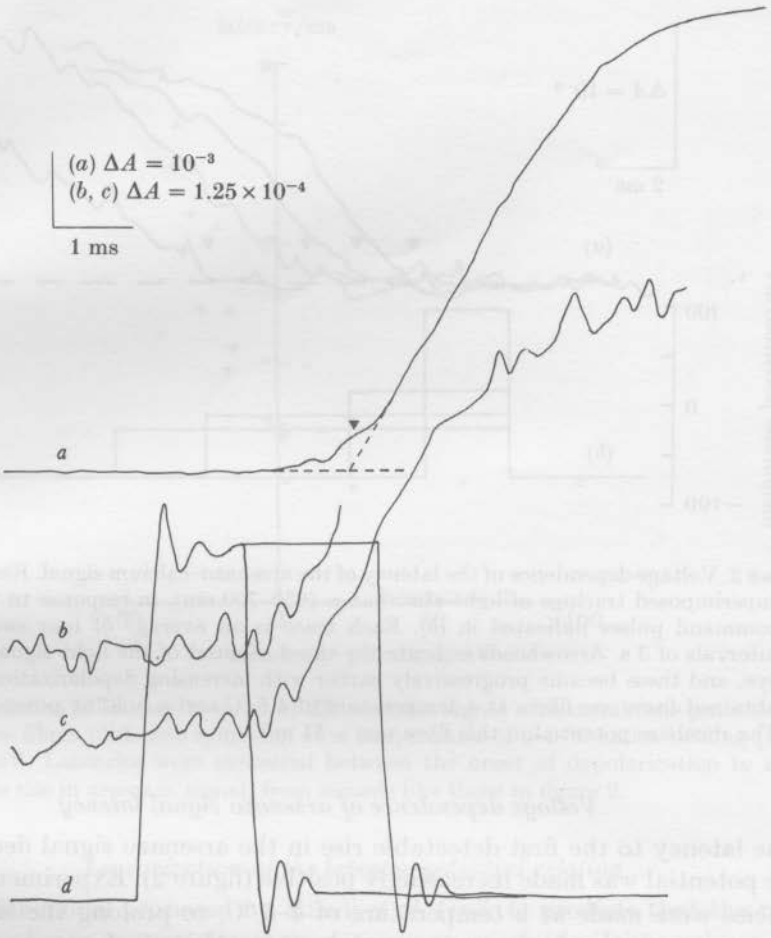

FIGURE 1. Latency of the arsenazo signal evoked by voltage clamp depolarizations. Traces (*a–c*) show absorbance changes at the wavelength pair 650–700 nm, and trace (*d*) shows superimposed records of the membrane potential. The calibration bar for the optical trace indicates the fractional change in absorbance ΔA. Temperature was 10 °C. (*a*) Response to a 3 ms depolarizing pulse from −75 to 0 mV, shown on a low recording gain. Average of eight sweeps obtained at 3 s intervals. Arrowhead indicates the latency estimated from the intersection of the baseline and tangent drawn to the main rising phase of the response. (*b*) The foot of the response in (*a*) shown at an eight times higher gain. (*c*) Response to a pulse of 1.3 ms duration to 0 mV. Same recording gain as (*b*). Average of 32 sweeps.

latency between the end of the pulse and the onset of the arsenazo response. Thus depolarization sets in motion a process of calcium release, which continues for several milliseconds after the depolarization has ceased. The second feature is that the initial phases of the arsenazo responses to the two pulses matched closely for about the first millisecond of their rise (figure 1*b,c*), even though the longer pulse gave a final response amplitude some eight times that of the shorter pulse. Thus the initial rise in intracellular calcium during the first millisecond of release was little affected by the membrane potential pertaining at that time, although it was, of course, a function of the pulse potential preceding the onset of the signal.

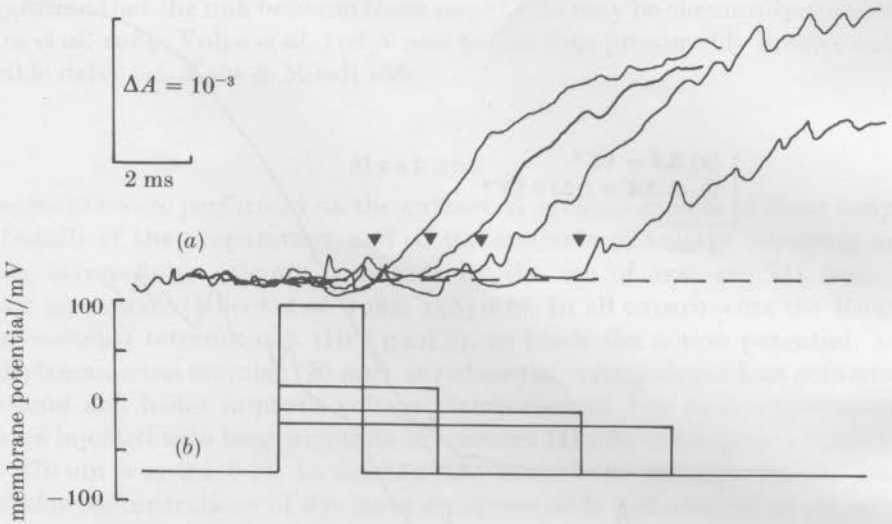

FIGURE 2. Voltage dependence of the latency of the arsenazo–calcium signal. Records in (*a*) show superimposed tracings of light absorbance (650–700 nm), in response to the depolarizing command pulses indicated in (*b*). Each trace is an average of four sweeps, elicited at intervals of 3 s. Arrowheads indicate the times of onset of the light signals, estimated by eye, and these became progressively earlier with increasing depolarization. Records were obtained from one fibre, at a temperature of 4.5 °C and a holding potential of −75 mV. The rheobase potential in this fibre was −51 mV.

Voltage dependence of arsenazo signal latency

The latency to the first detectable rise in the arsenazo signal decreased as the pulse potential was made increasingly positive (figure 2). Experiments to measure latencies were made at a temperature of 4–5 °C, to prolong the latency (Miledi *et al.* 1979) and thus facilitate its measurement. Also, the low temperature would reduce possible contributions from the settling time of the voltage clamp and the electrotonic time constant of the T-tubules. Estimates of the onset of the signal were made by eye from averaged traces of four or more sweeps. A pulse protocol was used whereby the pulse duration was progressively reduced as the pulse amplitude was increased, to give peak arsenazo signals of roughly similar amplitude (figure 2*b*). The selected amplitude was quite small, corresponding to about one fifth of the size that would have been elicited by an action potential (Miledi *et al.* 1982). This procedure had several advantages; (i) damage caused by residual contractions was minimized, (ii) the small calcium release allowed more frequent repetition of stimuli without 'fatiguing' the e.–c. coupling process (Miledi *et al.* 1983*a*), and (iii) any apparent change in signal latency resulting from changes in dye response with different amounts of calcium release would be minimized.

Figure 3 shows the relation between membrane potential and latency of the arsenazo signal, measured in three fibres at 4–5 °C from records like those in figure 2. The latency varied from about 10 ms at −40 mV to a minimum of about 2 ms at high positive potentials.

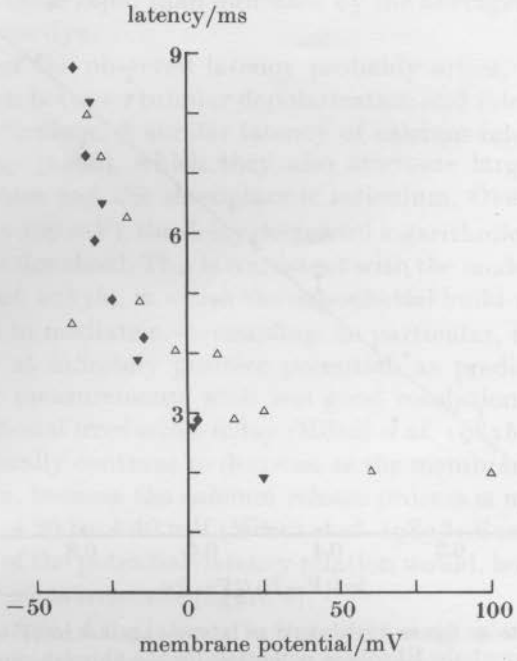

FIGURE 3. Variation in latency to onset of the arsenazo signal with membrane potential. Data from three fibres (different symbols), at a temperature of 4–5 °C and a holding potential of −75 mV. Latencies were measured between the onset of depolarization to the first detectable rise in arsenazo signal, from records like those in figure 2.

Linearization of the latency–potential relation

The coupler model proposed by Miledi *et al.* (1983*b*) predicts that the relation between membrane potential and the latency to onset of calcium release should follow the equation

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

where t = signal latency, τ = time constant for build up of coupler, H = holding potential, V = potential during pulse and R = rheobase potential for threshold calcium signal. To facilitate analysis we replotted the measurements, with t as ordinate and $\ln\{(V-H)/(V-R)\}$ as abscissa (see also Miledi *et al.* 1984). If the data points follow (1), then this choice of axes will give a straight line, which extrapolates to pass through the origin, and which has a slope (for an e-fold change in $(V-H)/(V-R)$) of τ.

The data of figure 3 have been replotted in this way in figure 4. The rheobase potential was measured for each fibre as the potential required during a 50 ms duration pulse to elicit a just-detectable arsenazo signal. The experimental points are fitted well by a linear regression line, which passes close to the origin, and has a slope corresponding to a value of τ of 8.7 ms.

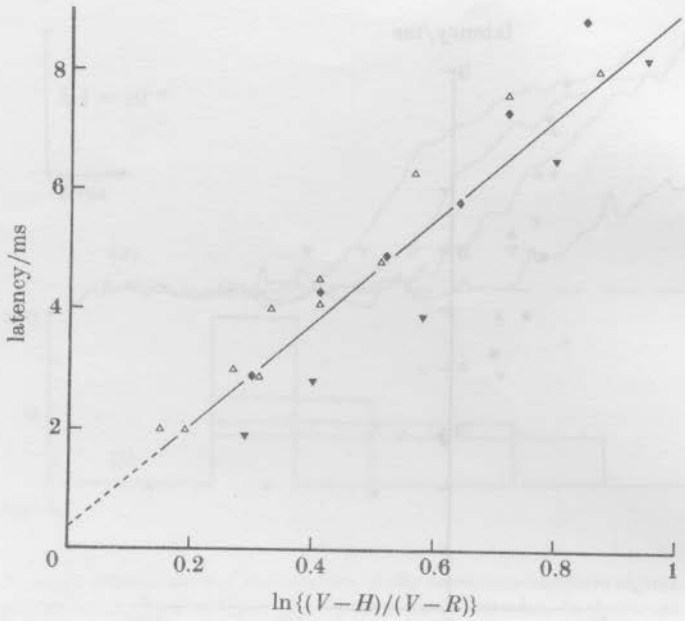

FIGURE 4. The same data as figure 3 replotted as latency against $\ln\{(V-H)/(V-R)\}$. See text for explanation of symbols. Rheobase potentials for the fibres shown were −52.6 mV (◆), −51.3 mV (△) and −48.4 mV (▼). Because of differences in the rheobase, points along the ordinate correspond to slightly different absolute membrane potentials for each fibre. As a rough guide, $\ln\{(V-H)/(V-R)\} = 0.2$ corresponds to about +100 mV, 0.5 to −20 mV and 1 to −40 mV.

DISCUSSION

The results show that there is a latency between the beginning of a depolarizing pulse and the first detectable rise in intracellular calcium as monitored with arsenazo III. This latency varies with the size of depolarization, and at 5 °C varies between about 8 ms at a membrane potential of −40 mV and 2 ms at positive potentials. Several factors might contribute to the latency. One is the reaction time of the indicator dye with calcium. However, this is probably rapid compared with the total delay, since the rise of the arsenazo signal during a twitch matches almost exactly that recorded with antipyrylazo III, a dye that is reckoned to have very rapid kinetics (Baylor *et al.* 1982). A second factor is the electrical time constant of the T-tubular system. The delay from this source is also likely to be short compared with the total latency (Miledi *et al.* 1983*b*). One argument is that the overall tubular time constant, estimated by using a potential sensitive dye in fibres similar to the ones in the present studies, was about 0.6 ms (Miledi *et al.* 1983*b*). For a depolarization to +100 mV, a time constant of this magnitude would introduce a delay of about 0.1 ms in charging the tubular potential to the threshold for calcium release (−50 mV) from a holding potential of −75 mV. Furthermore, the first release of intracellular calcium presumably occurs in areas of the fibre close to the surface membrane, where the depolarization of the

T-tubules would be more rapid than indicated by the averaged value provided by the potential sensitive dye.

The major part of the observed latency probably arises, therefore, from the stage in transmission between tubular depolarization and release of calcium from the sarcoplasmic reticulum. A similar latency of calcium release has been found by Vergara & Delay (1986), which they also attribute largely to transmission between the T-tubules and the sarcoplasmic reticulum. Over the voltage range examined (−40 to +100 mV), the delay decreased logarithmically as the potential was increased above threshold. This is consistent with the model we had previously proposed (Miledi *et al.* 1983*b*), in which the exponential build-up of a hypothetical coupler is supposed to mediate e.–c. coupling. In particular, the latency extrapolated close to zero at infinitely positive potential, as predicted by the model, whereas our earlier measurements with less good resolution had suggested the presence of an additional irreducible delay (Miledi *et al.* 1983*b*). It is unlikely that the latency does actually continue to decrease as the membrane potential is made increasingly positive, because the calcium release process is maximally activated at potentials above +20 to +40 mV (Miledi *et al.* 1983*b*; Eusebi *et al.* 1983). The expected flattening of the potential–latency relation would, however, be too slight to be seen from our measurements (figure 4).

An interesting feature of the latency in the calcium signal is that it was evident both when the fibre was depolarized and when it was subsequently hyperpolarized. That is to say, there was a latency between depolarization and the onset of calcium release, and then when the fibre was returned to the holding potential a similar latency was seen before the rate of rise of the calcium signal began to decline.

The minimal latency of the arsenazo signal in our recordings was 2 ms (at 4–5 °C and +20 to +100 mV), and as discussed above it probably arose mainly at the stage between the depolarization of the T-tubules and calcium release from the sarcoplasmic reticulum. This delay is sufficiently long to be compatible with the hypothesis that e.–c. coupling may be mediated chemically via inositol phospholipid metabolism (Vergara *et al.* 1985; Volpe *et al.* 1985). Further evidence supporting the idea of chemically mediated e.–c. coupling comes from the large temperature dependence of the latency between the rise of the action potential and the onset of the arsenazo signal. This shows a Q_{10} of 2.5 between 5 and 15 °C (Miledi *et al.* 1979), which is typical of an enzyme-mediated reaction. In contrast, if electrotonic depolarization of the T-tubules were responsible for the delay, a diffusion-limited Q_{10} of about 1.3 might be expected.

Some instructive comparisons can be made between the e.–c. coupling mechanism in muscle and the process of synaptic transmission at the neuromuscular junction, which is a well studied example of chemical transmission. Synaptic transmission involves a similar minimal latency (2–3 ms at 5 °C), and shows a high temperature dependence (Q_{10} of about 3) (Katz & Miledi 1965). However, the mechanisms involved in these two processes are quite different and lead to some differences in properties. Depolarization of the nerve terminal directly activates membrane calcium channels, resulting in a calcium influx that subsequently triggers the release of transmitter. When the membrane potential is made strongly positive, transmitter release is suppressed because of the reduced driving force for calcium

influx, and there is a 'latency shift' so that release is delayed until the end of the pulse (Katz & Miledi 1967). In e.–c. coupling, calcium release from the sarcoplasmic reticulum is linked to depolarization by an intermediate mechanism. The calcium release is maximal with depolarization to around 0 mV but, differently from synaptic transmission, polarization beyond this voltage does not reduce the size of the calcium signal (Miledi *et al.* 1977, 1983*b*; Eusebi *et al.* 1984). We also found no evidence for a latency shift with strong depolarization. At high positive potentials the onset of the calcium–arsenazo signal was not delayed until the end of the pulse, but instead occurred during the pulse with a latency similar to, or shorter than, that with smaller depolarizations (cf. figure 1).

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