Birefringence signals and tension development in single frog muscle fibres at short stimulus intervals

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Summary. The early large birefringence signal and mechanical activity were studied together in isolated single fibres of frog skeletal muscle with double stimulation at short stimulus intervals (2–60 msec) at room temperature and at 4–6 °C. In all fibres tested, extra tension and additional birefringence signal in response to the second stimulus appeared simultaneously and suddenly upon increasing the stimulus interval. The shape of the stimulus-interval versus tension-development curve makes it highly improbable that subthreshold calcium release occurs at shorter stimulus intervals; therefore, tension development reliably reflects Ca-release in these experiments. In contrast to the report by Suarez-Kurtz and Parker, birefringence signal and calcium release are shown not to be dissociated by double stimulation. This result supports the hypothesis that the early large birefringence signal is an intrinsic indicator of calcium release from the sr during EC-coupling in skeletal muscle.

During activation of single frog muscle fibres in hypertonic Ringer a 3-component birefringence transient is observed[1,2]. The small 1st component reflects the surface action potential and is immediately followed by the early large signal (2nd component). In normal Ringer the 1st component is lost in the falling phase of the early large signal which clearly precedes latency relaxation at all tonicities tested and is followed by a transient apparently related to tension development (3rd component). Therefore, the early large signal probably reflects a step in excitation contraction coupling prior to the activation of the contractile proteins. This hypothesis can only be correct if every excitation leading to a contractile response elicits a birefringence signal.

Recently, evidence against this hypothesis has been reported by Suarez-Kurtz and Parker[3] based on double stimulation experiments with whole cutaneous pectoris muscle at low temperature. The 2nd stimulus elicited a birefringence signal only if the stimulus interval was longer than 40 msec, whereas a calcium transient could be demonstrated with Arsenazo III down to a stimulus interval of 20 msec (Miledi and Schalow, unpublished results, quoted by Suarez-Kurtz and Parker[3]). These data, suggesting the possibility of calcium release without a birefringence signal, contradicted our own unpublished results with double stimulation at room temperature. We had always found a strict correlation between the appearance of extra tension and an additional birefringence signal in response to the 2nd stimulus. We therefore repeated these experiments at room temperature as well as in the cold (4–6 °C).

Single muscle fibres isolated from the M.iliofibularis of *Rana temporaria* were mounted as described earlier[2]. They were placed between crossed polarizers and illuminated with monochromatic light (624 nm). Light intensity changes, caused by changes in optical retardation of the muscle fibre, were measured with a photodiode (EG & G, UV 444 B). The fibres were stimulated externally with 0.2-msec suprathreshold pulses and were stretched to about 180% slack length, until the early large birefringence transient was well separated from the later component, but twitch tension, measured with an RCA 5734 transducer, remained high enough to indicate calcium release. Stimulation alternated between a single stimulus and double stimulation with a given stimulus interval. The tension- and light-intensity-signals were averaged separately for the two stimulus protocols in a 4-trace Nicolet signal averager with the possibility to obtain differences between traces. After control measurements at room temperature the whole preparation was cooled down to 4–6 °C in a refrigerator equipped with an optical window to permit continuation of the measurements in the cold. The temperature of the bath containing the muscle fibre was held constant to within ±0.2 °C during the course of an experiment and was monitored with a calibrated Philips NTC thermistor.

In figure 1 the averaged responses to a single stimulus and to double stimulation at a stimulus interval of 40 msec are compared at 22 °C (A) and at 4 °C (B). At room temperature birefringence signal and extra tension in response to the 2nd stimulus are clearly visible, whereas in the cold only extra tension is obvious. If, however, the response to a single stimulus is now subtracted from the double stimulus trace in the signal averager memory, this difference reflects the fibre response to the 2nd stimulus alone. Sufficiently enlarged an early birefringence signal (amplitude less than 2% of the original signal) can be detected in the cold as well.

In figure 2 extra tension and birefringence signals are shown at 3.9-, 4-, 7-, 10-, and 25-msec intervals at 22 °C. At a stimulus interval of 3.9 msec no optical or mechanical response to the 2nd stimulus can be detected. Within 0.1-

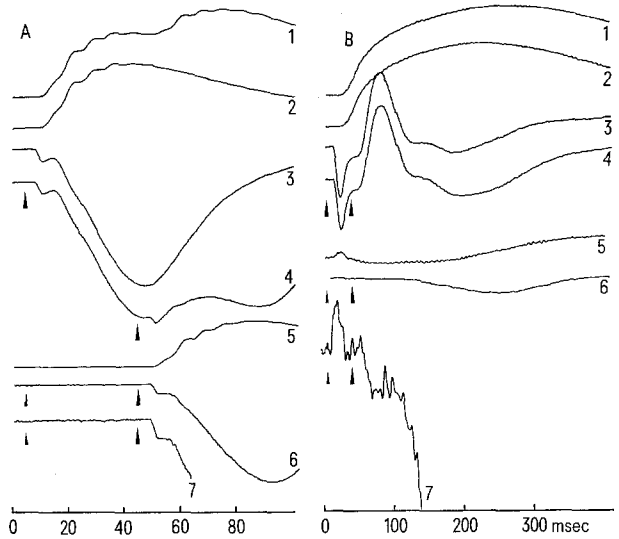

Fig. 1. Determination of response to the second stimulus by subtraction in double stimulation experiments. Stimulus interval 40 msec. *A* at 22 °C, *B* at 4 °C. Note the difference in time scale. Arrows indicate stimulation (0.2-msec pulse). Top group of traces (1–4): Response to double stimulation (1 tension, 4 birefrengence) compared to a single stimulus (2 tension, 3 birefringence). Middle pair of traces (5, 6): Response to the 2nd stimulus alone obtained by subtraction. Tension (5) = trace 1 – trace 2, birefringence signal (6) = trace 4 – trace 3. Bottom trace (7): Birefringence signal (6) at higher gain to bring the signal approximately to the size of the single stimulus response. 7_A enlarged 2×, 7_B enlarged 40×. Despite the low signal to noise ratio the birefringence signal in 7_B can be clearly identified due to its correct delay with respect to the 2nd stimulus. The signal is shifted by 20 msec to the right if the stimulus interval is increased to 60 msec, it disappears if the interval is decreased to 35 msec. Simultaneously, extra tension disappears completely in the latter case.

msec stimulus interval the birefringence signal jumps from 0 to 17% and extra tension simultaneously rises from 0 to 23% of the original amplitude. In figure 3 tension and birefringence signal amplitude are plotted versus log stimulus interval for a typical fibre at room temperature and at 4 °C. In both cases, tension and birefringence signal appear suddenly and simultaneously (within 0.1 msec at 22 °C, within 1 msec at 4 °C) as in all fibres tested (room temperature 8 fibres, cold 4 fibres). At room temperature threshold stimulus interval ranged from 3 to 5 msec, in the cold the range was 20–60 msec. After the sudden onset, birefringence signal amplitude rises smoothly over about 1 decade stimulus interval, usually with some indication of a plateau at short intervals, particularly in the cold. This plateau is more prominent in the recovery of twitch tension which usually overshoots at about 10-fold threshold stimulus interval and then gradually declines to 100%.

The main effect of lowering the temperature by 16–18 °C is clearly a slowdown of signal- and tension-recovery by about a factor of 10, corresponding to a Q_{10} of approximately 4. In addition, cooling reduces the response amplitude to the 2nd stimulus at intervals near threshold (birefringence signal reduced to 10–20%, tension to 30–50% compared with near threshold interval responses at room temperature). The response to the 1st stimulus is much less affected by cooling (the birefringence amplitude is reduced by about 20%, the twitch is potentiated to 120–180%).

The main result of the double stimulation experiments reported here is the strict coupling of birefringence signal and Ca-release as measured by the development of tension. In no case could we detect any trace of extra tension at short intervals without a birefringence signal in response to the 2nd stimulus. One could argue that at very short stimulus intervals Ca-release is below the threshold for contraction. This seems highly improbable because of the sudden collapse of tension from a level of about one third of twitch tension to no tension at all within a fraction of a msec, when the stimulus interval is progressively shortened. If Ca-release would decrease below the threshold for activation of the contractile proteins with decreasing stimulus interval we would expect an s-shaped decline of tension, as indicated by a log [Ca]-versus-tension plot[4,5]. The sudden collapse of tension as in our figure 3 is much more likely caused by a process prior to Ca-release being refractory, either surface action potential or the processes underlying charge movement[6]. Our conclusion is therefore, that in double stimulation experiments, where the sequence of EC-coupling has to be initiated by an action potential, subthreshold Ca-release does not occur. So far it has been demonstrated in voltage clamp experiments only[7].

The opposite results by Suarez-Kurtz and Parker[3] can be explained in 2 ways: a) The critical birefringence measurements and the Ca-release experiments with Arsenazo III were not performed on the same preparations (the Arsenazo results are not shown, but only reported as 'Miledi and Schalow, unpublished data'). The threshold stimulus interval is highly variable in the cold, ranging from 20 to 60 msec in our experiments at 4 °C. Even at the same temperature it is therefore possible to measure Ca-release in one fibre and not to detect a birefringence signal in another one. b) As seen from the records at 4 °C (figure 1, B_3 and B_4) it is quite difficult to determine the exact time course of extra signal recovery at intervals near threshold without using a subtraction method as described above. Actually, if the double stimulus record at 35-msec stimulus interval in figure 3a by Suarez-Kurtz and Parker[3] is compared with their single stimulus response by tracing one of the signals on a piece of transparent paper and superimposing the rising phases of the transients due to the 1st

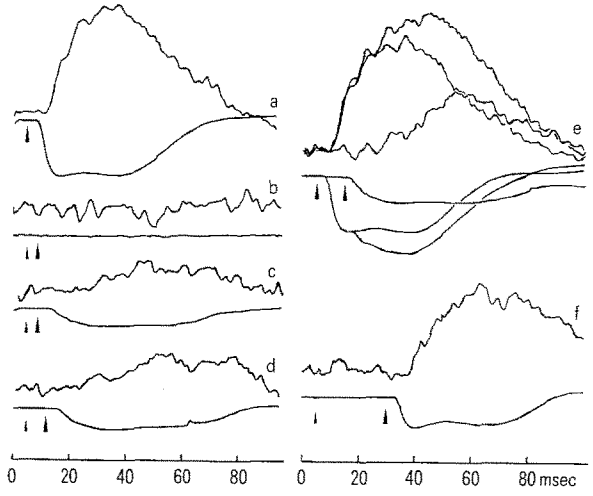

Fig. 2. Recovery of birefringence signal (lower traces) and tension (upper traces). *a* Response to a single stimulus; *b–f* response to 2nd stimulus obtained by subtraction; *b* no response at a stimulus interval of 3.9 msec. *c–f* Increasing tension- and birefringence signals at intervals of 4 msec (*c*), 7 msec (*d*), 10 msec (*e*), 25 msec (*f*). In *e* the response to a single stimulus, to double stimulation and the response to the 2nd stimulus alone, obtained by subtraction, are presented. Note summation of the birefringence signals. Large arrows indicate stimulus which produced the response shown, small arrows indicate the time of the 1st stimulus, where the traces are the result of subtraction (see figure 1).

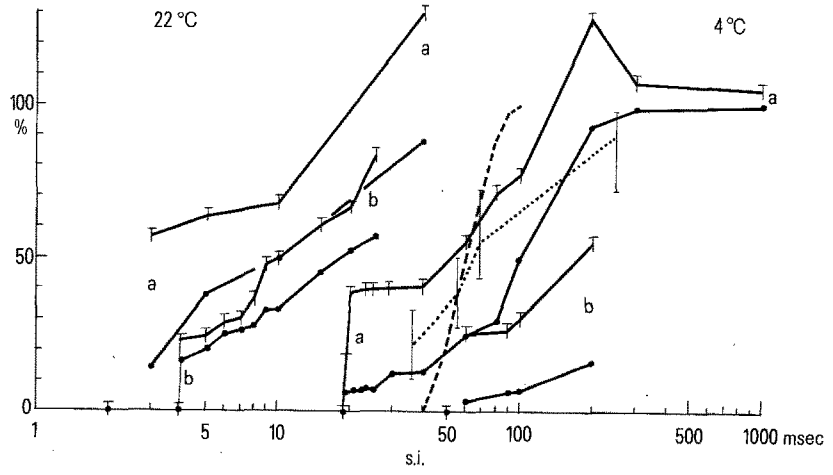

Fig. 3. Birefringence signal (●)- and tension (T)-recovery (%, ordinate) versus stimulus interval (msec, abscissa) at 22 °C (left) and at 4 °C (right). Fibre *a* accurate determination of threshold interval at 4 °C. At 22 °C threshold between 2 and 3 msec (not connected). Fibre *b* accurate determination of threshold interval at 22 °C. At 4 °C threshold between 50 and 60 msec (not connected). The dashed curve indicates signal recovery given by Suarez-Kurtz and Parker[3] in their figure 3b. The dotted line corresponds to our tentative measurements taken from their original traces in figure 3a, see text. The vertical bars represent the approximate range of this estimation.

stimulus, a small birefringence signal in response to the 2nd stimulus can be detected. Once the difference has been spotted the convex falling phase and the increased undershoot of the double stimulus response (bottom) compared to the single stimulus trace (top) are quite prominent. A tentative analysis of the full set of original traces in their figure 3a seems to indicate fair agreement of these values with our own data (see our figure 3).

Suarez-Kurtz and Parker[3] suggest that the early birefringence signal is caused by a process which is fully saturated during a twitch, like Ca-binding to troponin or latency relaxation. Under favourable conditions, however, the birefringence transients do summate (see figure 2e), they clearly precede latency releaxation[2,8], and birefringence signal and latency relaxation can be dissociated[9].

Therefore, in contrast to the interpretation presented by Suarez-Kurtz and Parker[3], we conclude that the results of double stimulation experiments do not disprove the hypothesis that the early large birefringence signal reflects a potential change of the sr membrane in response to the release of Ca from the sr. This hypothesis has been proposed[1] because potential changes across excitable membranes (muscle surface membrane[1,2], pike olfactory nerve[10], squid giant axon[11]) are known to produce corresponding birefringence signals and because muscle fibres stained with a fluorescent dye known to monitor membrane potential in nerve (indodicarbocyanine) produce a fluorescent transient with the same time course as the early large birefringence signal[12].

The observation that the signal caused by a 2nd stimulus at short intervals is too small to produce an obvious increase in total signal size (figure 1) is in agreement with the assumption that the longitudinal sr is calcium sensitive and undergoes a Nernst-potential change in response to the rapid change in myoplasmic $[Ca^{++}]$. The additional potential change in response to an immediately following 2nd release of Ca^{++} is then expected to be much smaller even if the same amount of Ca^{++} would be released which is probably not the case. This alternative model predicts the observed signal size better than the original assumption that the Ca-current is charging the membrane capacitance[1], where a potential change proportional to the amount of Ca^{++} released would be expected.

If a calcium sensitive longitudinal sr membrane is assumed to cause the signal, injection of EGTA into a muscle fibre would abolish the Nernst-potential change by preventing the rise in free myoplasmic $[Ca^{++}]$, in agreement with the observations by Suarez-Kurtz and Parker[3]. As these authors point out, a signal directly caused by the Ca-current charging the sr membrane should have remained unaffected, provided that EGTA does not interfere with the Ca-release mechanism.

In conclusion, therefore, the results of these series of experiments with double stimulation indicate that a change in sr potential has remained a likely candidate for the unknown process during ec-coupling causing the early large birefringence signal in skeletal muscle fibres.

1 S.M. Baylor and H. Oetliker, Nature *253*, 91 (1975).
2 S.M. Baylor and H. Oetliker, J. Physiol., Lond. *264*, 141 (1977).
3 G. Suarez-Kurtz and I. Parker, Nature *270*, 746 (1977).
4 W. Almers and P.M. Best, J. Physiol., Lond. *262*, 583 (1976).
5 D.C. Hellam and R.J. Podolsky, J. Physiol., Lond. *200*, 807 (1969).
6 M.F. Schneider and W.K. Chandler, Nature *242*, 244 (1973).
7 R.H. Adrian, W.K. Chandler and A.L. Hodgkin, J. Physiol., Lond. *204*, 207 (1969).
8 P. Haugen and O. Sten-Knudsen, J. gen. Physiol. *68*, 247 (1976).
9 H. Oetliker and R.A. Schümperli, Experientia *34*, 902 (1978).
10 A. v. Muralt, E.R. Weibel and J.V. Howarth, Pflügers Arch. Physiol. *367*, 67 (1976).
11 L.B. Cohen, B. Hille and R.D. Keynes, J. Physiol., Lond. *211*, 495 (1970).
12 H. Oetliker, S.M. Baylor and W.K. Chandler, Nature *257*, 693 (1975).

Thiamin turnover rate in some areas of rat brain and liver: A preliminary note[1]

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Summary. Thiamin turnover rates in some nervous structures and liver of rats were evaluated in a steady state condition, using thiamin-^{14}C as a tracer. The radioactivity contents were analyzed by means of a mamillary type compartmental model. Excluding the liver, turnover rate values of the nervous structures were ordered in the following sequence: sciatic nerve (0.58 μg/g · h) > cerebellum > hypothalamus > midbrain > corpus striatum > cerebral cortex (0.16 μg/g · h).

The problem of thiamin function in the nervous system (coenzymatic and/or noncoenzymatic) is yet unresolved[2]. A useful approach might be a comparative study of thiamin turnover in brain areas, particularly those selectively affected by thiamin-deficiency[3]. This prompted us to perform an investigation on thiamin turnover in the nervous system, since no data are available in the literature. Here we will briefly give the results obtained on the rat brain, using labelled thiamin as a tracer in a steady-state condition. The liver was also included for comparison purposes, because of its thiamin storage function in the body. A complete account will be published elsewhere.

Materials and methods. 30 μg of [Thiazole-2-^{14}C]-thiamin (Radiochemical Center, Amersham, England: specific activity 14 mCi/mmole) were i.p. injected into female Wistar rats (230–280 g of b.wt), starved at night. Since the amount of thiamin injected corresponds to the rat's daily requirement[4], during the successive 24 h the rats were fed a thiamin-deficient diet (Dr Piccioni, Brescia, Italy), and then again a complete diet. In this way, thiamin intake and total thiamin organ contents were kept constant (steady state condition). The rats were sacrificed by decapitation at time intervals from 5 min to 96 h from thiamin injection. Different nervous structures (table) and a sample of liver were rapidly dissected in the cold. For brain dissection, the method of Glowinski and Iversen[5] was followed. Thiamin was extracted from the tissues with cold 0.5 N HCl, and 40% trichloroacetic acid was used for deproteinization. Labelled free and phosphorylated thiamin of the extracts were separated following Sharma and Quastel[6]. Thiamin radioactivity was determined by a Geiger-Müller low background flow-counter (Nuclear Chicago, mod. 512) after drying suitable samples on planchets. The efficiency of the counting was 81% and the error was less than 2%. Total (sum of free and phosphorylated) labelled thiamin radioactivity was expressed as nCi/g of wet tissue. Preliminary experiments showed a mean recovery of the labelled thiamin added to tissue samples of 97% (10 experiments). The