Histochemical Study of Intracellular Calcium Shifts During Activation of Contraction in Crayfish Muscle Fibres

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Abstract. Intracellular calcium shifts were studied by means of the lead acetate method in crayfish muscle fibres activated either by caffeine or by membrane depolarisation (tetanic and potassium conctracture). Caffeine activation resulted in a marked depletion of calcium stores localised in the sarcoplasmic reticulum (SR) whereas fibres activated by potassium depolarisation exhibited a pronounced increase in the myofibrillar calcium. The shifts of specific precipitates from and to the SR were fairly uniform including all SR elements along the sarcomere. The observations support the hypothesis on partially different sources of activating calcium for contractions elicited by membrane depolarisation and caffeine respectively in crayfish muscle fibres.

Key words: Crayfish muscle — Sarcoplasmic reticulum — Intracellular calcium translocation — Activation of contraction — Electron microscopic histochemistry

Introduction

It is generally accepted that calcium plays an important role in the contraction-relaxation cycle of muscle cells (Zachar 1971).

The capacity of sarcoplasmic reticulum (SR) vesicles to accumulate calcium was demonstrated both in microsomal fraction (Ebashi 1961; Hasselbach and Makinose 1961) and *in situ* using histochemical (Costantin et al. 1965; Zebe and Hasselbach 1966) as well as X-ray microanalytical methods (Somlyo et al. 1977).

A logical extension of these investigations has been the attempt to demonstrate intracellular calcium shifts during the contraction-relaxation cycle using morphological techniques. As contrasted with the numerous papers dealing with activity dependent calcium translocations in smooth muscle cells (Atsumi and Sugi 1976; Sugi and Daimon 1977; Atsumi 1978; Suzuki and Sugi 1978; Fukuoka et al. 1980; Rubányi et al. 1980; Sugi et al. 1981, 1982; Suzuki 1982) relatively few studies were conducted on skeletal muscle cells. Using the method of autoradio-

graphy Winegrad (1965, 1968, 1970) could show that during tetanic stimulation calcium was displaced from the level of the Z-lines (where the terminal cisternae are located) to the site of the myosin/actin interaction; a reverse shift could be observed during relaxation. Under similar conditions but using the pyroantimonate method (Yarom and Meiri 1971; Samosudova and Lyudkovskaya 1979) no changes in Ca-precipitates contained in terminal cisternae (TC) were observed; instead, the precipitates located in I-bands were dispersed along the myofilaments (Yarom and Meiri 1971). An unaltered density of pyroantimonate precipitates in TC was reported by McCallister and Hadek (1973) even in muscle fibres treated repeatedly by caffeine in a Ca-free solution.

Different results were obtained by Somlyo et al. (1981) on tetanized frog muscles using electron-probe analysis of ultrathin cryosections: 59% of the calcium content of TC was released thus suggesting that TC are the major site of Ca storage and release in the contraction-relaxation cycle.

Due to differences and discrepancies in the above-mentioned studies further work is needed to clarify the question of intracellular Ca translocation in skeletal muscle cells.

In the present work histochemical methods were used to demonstrate intracellular calcium distribution in crayfish muscle fibres during activation of contraction induced either by caffeine or by depolarisation of the cell membrane. The crayfish muscle seems to be of special interest for such comparative investigations due to clear-cut differences in the dependence upon the extracellular calcium between caffeine-induced and depolarisation-evoked contractile responses (Zachar 1971). Our results revealed that there are corresponding differences in the involvement of the calcium from the SR between the two types of contractile activation. In crayfish muscle fibres the shift of precipitates from and to SR is fairly uniform including all SR elements along the sarcomere.

Some of the results were reported briefly elsewhere (Uhrík and Zacharová 1981).

Material and Methods

Single muscle fibres from m. extensor carpopoditi of the crayfish (*Astacus fluviatilis*) were dissected according to Zachar et al. (1964) and equilibrated in crayfish saline containing (in mmol/l): Na^+ (205.3); K^+ (5.4); Ca^{2+} (13.5); Mg^{2+} (5.6); Cl^- (248.8); $TRIS^+$ (5); pH was adjusted to 7.3—7.5. Potassium contractures were induced by sudden exchange of the normal crayfish saline for a saline with an increased potassium concentration (43.2 mmol/l K^+) at a constant $[K]_o \times [Cl]_o$. The procedure was described elsewhere (Zachar and Zacharová 1966).

Tetanic stimulation (40 Hz) was applied by means of two platinum plate electrodes (massive stimulation). Caffeine was dissolved directly in the crayfish saline to give the final concentration of 6 mmol/l. The RCA 5734 transducer was used to record isometric tension.

The lead acetate method (Carasso and Favard 1966; Komnick 1970) was used for calcium demonstration. Fibres were fixed with 2% glutaraldehyde in 0.2 mmol/l phosphate buffer (pH 8). In

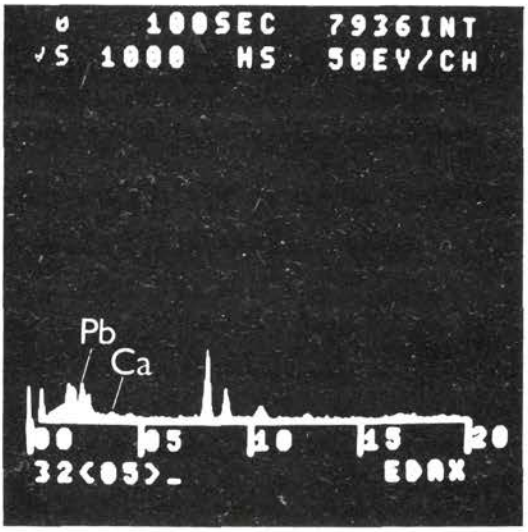

Fig. 1. X-ray spectrum from a precipitate in SR following the lead acetate histochemical procedure in a crayfish muscle fibre. 0.5 μm unstained section was used and the signal was collected for 100 sec at 40 kV.

the following steps lead was substituted for calcium in the phosphate to form electron-dense precipitates. Further details of the procedure are given in another paper (Uhrík and Zacharová 1979) which also demonstrates the usefulness of the method for contrasting SR in crayfish muscle fibres. The specificity of the method was confirmed on heart muscle by Diculescu et al. (1971) using EGTA as calcium chelating agent.

Ultrastructural appearance of activated fibres was compared with controls taken from the same animal and processed in parallel under otherwise identical conditions to exclude unspecific variations in myofibrillar precipitates.

After embedding in Epon 812 ultrathin (60—80 nm) sections were studied in a Tesla BS 613 electron microscope at 80 kV. Thick (2 μm) sections were examined in the JEM 1000 high voltage electron microscope at 1000 kV.

A preliminary study of the composition of the precipitates using energy dispersive X-ray analysis (EDAX 711 in connection with the microscope JEM 100 C) showed a large peak for the Pb M_{α} line (2345 eV) and a slight indication for the Ca K_{α} line (3691 eV) suggesting a practically complete substitution of lead for calcium in the fibre (Fig. 1).

Results

Normal appearance

Specific precipitates may be observed in SR vesicles, mitochondria and external (basal) lamina of sarcolemmal invaginations (clefts) as described previously (Uhrík and Zacharová 1979).

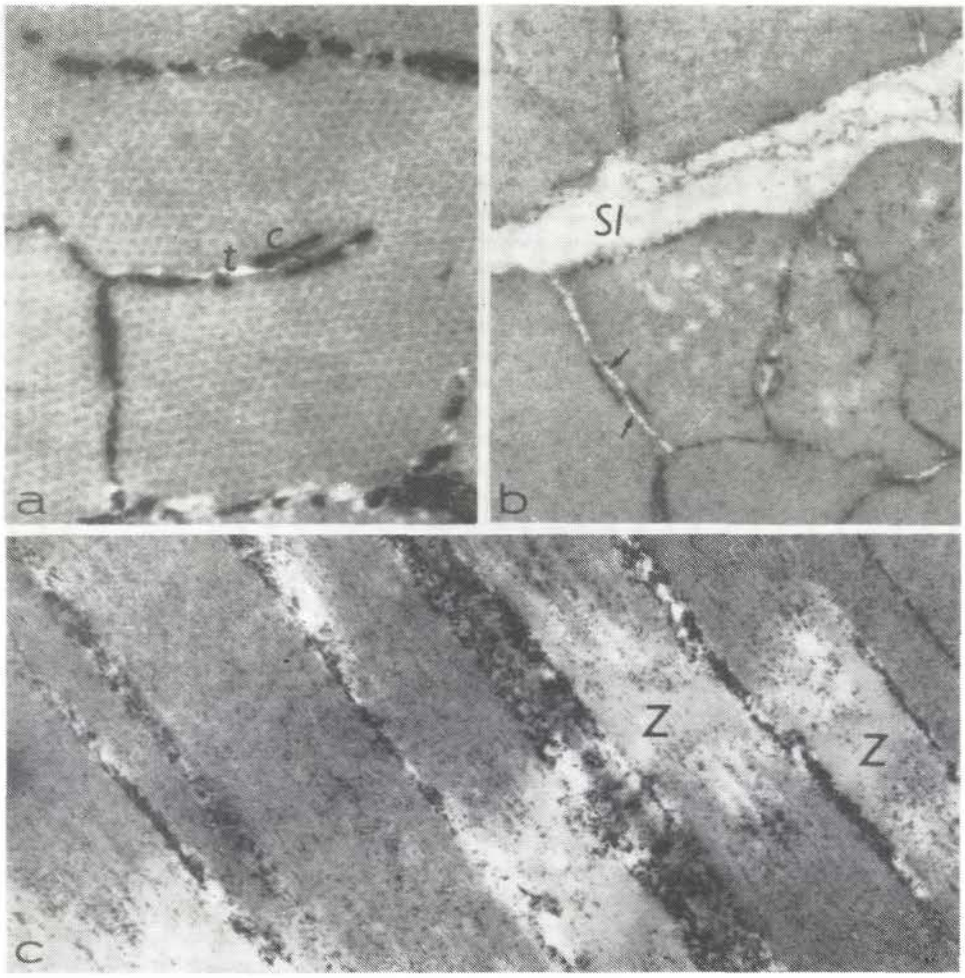

Fig. 2. Ultrathin sections. The distribution of the precipitates in crayfish muscle fibres in relaxed state. (*a*), (*b*) — transverse section, (*c*) — longitudinal section. The arrows in (*b*) point to fine precipitates along the membranes of a T-tubule. *t* — tubule of the T-system; *c* — cisterna of the SR; SI — sarcolemmal invagination; Z — Z-line. Magnification: × 53,700 (*a*), × 23,100 (*b*), × 23,800 (*c*).

Fig. 2 shows the appearance of crayfish muscle fibres processed with the lead acetate method in relaxed state. Fig. 2*a* and 2*b* differ in the amount of precipitates in the myofibrillar region, the fibre in (*a*) being virtually free from the precipitates. It was not clear why the fibres differed in this respect; some observations suggest that individual and seasonal differences might be responsible. These myofibrillar precipitates, if present, were diffusely dispersed between myofilaments with the highest density at the I band level and A/I boundary (Fig. 2*c*). An invariable

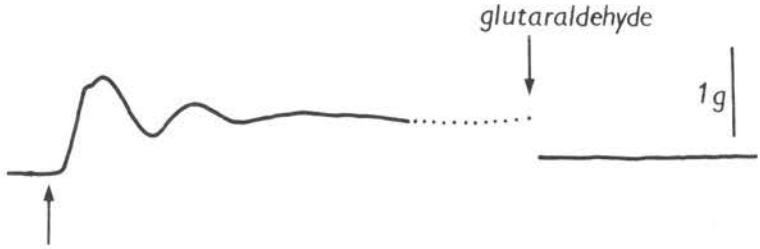

Fig. 3. Caffeine contracture in a single muscle fibre of the crayfish. 6 mmol/l caffeine (left arrow) was applied for 60 sec.

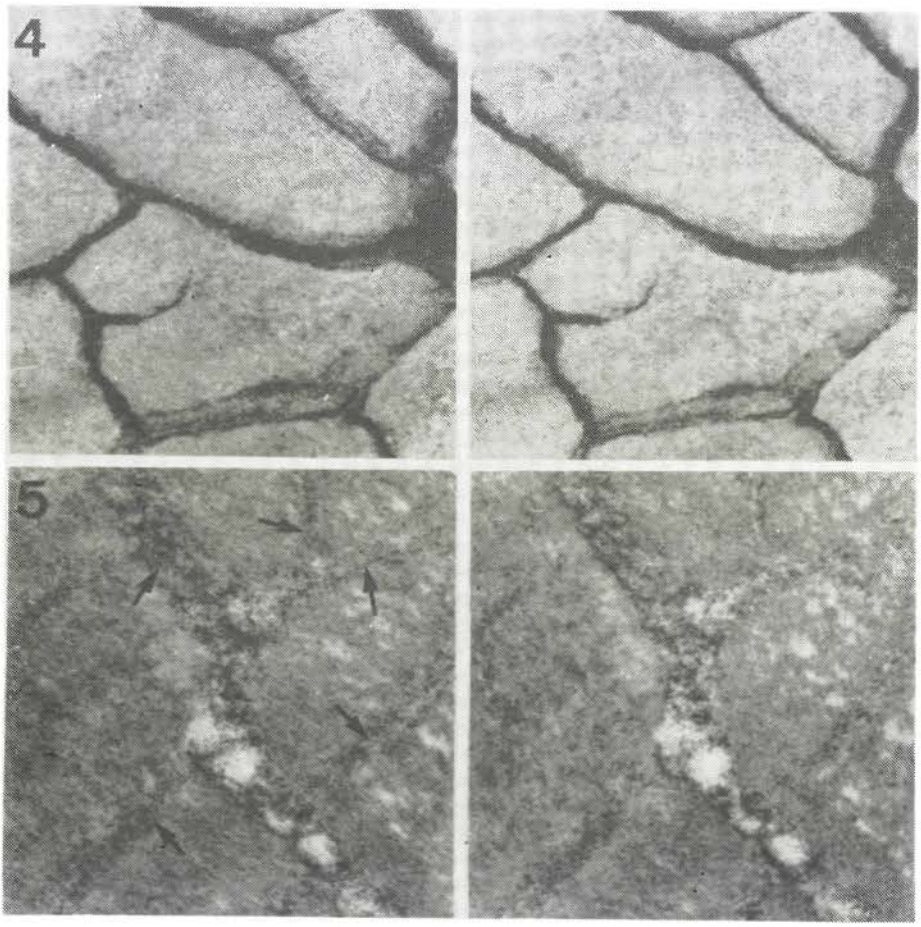

Fig. 4, 5. Stereo-pairs of transverse 2 μm thick sections in a high voltage electron microscope from the fibre in relaxed state (Fig. 4) and the caffeine-treated fibre (Fig. 5). The arrows in Fig. 5 point to boundaries (formed by SR elements) between individual myofibrils. Tilt: 0° and 4°. × 25,000.

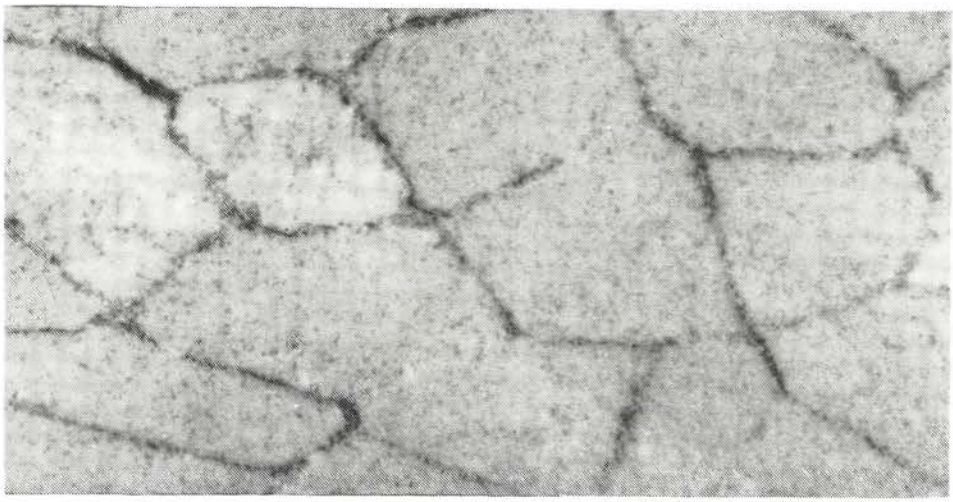

Fig. 6. Ultrathin transverse section of the fibre let to recover from the caffeine contracture for 30 min. *Calcium returned to the SR elements resulting in the distinct delineation of myofibrils.* × 23,100.

feature was the presence of heavy precipitates in all SR vesicles. The lumen of T-tubules was either empty (Fig. 2*a*) or contained fine precipitates along the tubular membranes (Fig. 2*b*).

Caffeine activation

Four pairs of fibres (test and control) were examined. In two cases the caffeine administration was preceded by a 6 min perfusion with Ca-free saline. No differences either in mechanical response or ultrastructural appearance were observed as compared with fibres perfused in normal saline prior to caffeine administration.

Fibres were exposed to caffeine for 60 s and then fixed for ultrastructural examination. The time course of caffeine contractures was generally the same as described previously (Uhrík and Zacharová 1976) with several peaks and a tonic plateau, as shown in Fig. 3.

The use of thick sections and their examination in a high voltage electron microscope may be advantageous for a more representative view of the distribution of precipitates. Fig. 4 shows a 2 μm thick section of a control fibre with a normal distribution of SR precipitates distinctly delineating individual myofibrils. In caffeine treated fibres the intensity of SR precipitates was decreased resulting in a poor delineation of myofibrils, whereas a moderate increase in the precipitate density was encountered in myofibrillar spaces (Fig. 5).

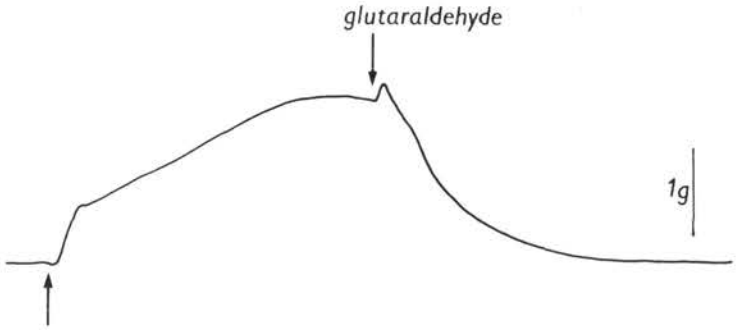

Fig. 7. Potassium contracture in a single muscle fibre of the crayfish. 43.2 mmol/l K^+ (left arrow) was applied for 25 sec.

These changes were reversible as demonstrated on the fibre recovering from the caffeine contracture for 30 min in normal saline (Fig. 6). Calcium was present in all elements of the sarcoplasmic reticulum along the sarcomere.

Depolarisation-induced activation

Four pairs of fibres were examined. In two cases the membrane depolarisation was induced by increasing the $[K]_o$ for 20 or 25 sec (Fig. 7); in two other cases the fibres were depolarised by 35 sec tetanic stimulation.

Fig. 8 demonstrates ultrastructural changes in a 2 μm section. In comparison with the normal appearance (Fig. 4) the distribution of precipitates during the potassium contracture shows a moderate decrease in intensity of SR precipitates and an overall increase in the precipitate density in the myofibrillar space with the same preference for I bands and A/I boundaries as seen in the control (Fig. 2*c*). In some regions the decrease in precipitate density in SR was more pronounced but has never reached the caffeine-induced decrease. On the other hand, the precipitate density in myofibrillar region was markedly increased in comparison with that during the caffeine contracture. There was no evidence for a different reactivity between the different parts of the SR along the sarcomere.

The fibres activated by tetanic stimulation for 35 sec did not differ significantly in the distribution and density of precipitates from controls (Fig. 9*a*, *b*).

Discussion

Single muscle fibre of the crayfish may be considered a preparation especially suitable for the application of histochemical precipitation methods since isolation

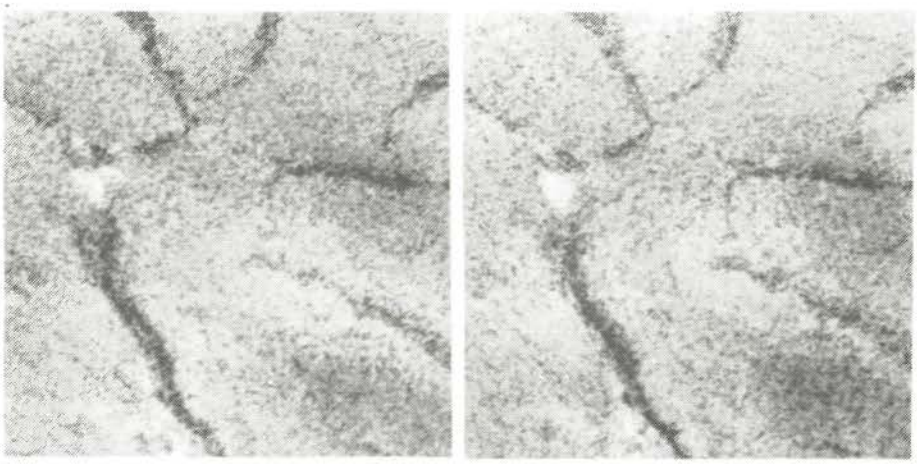

Fig. 8. Stereo-pair of a transverse 2 μm thick section in a high voltage electron microscope from the fibre undergoing potassium contracture. Note the marked increase in myofibrillar precipitates. Tilt: 0° and 4°. × 25,000.

from adjacent tissue and the presence of broad sarcolemmal invaginations or clefts (Uhrík et al. 1980) facilitate the diffusion of precipitating ions.

Previous physiological studies (Zacharová and Zachar 1967; Zachar 1971) have shown that potassium contractures in the crayfish muscle fibres are dependent on the presence of calcium ions in the extracellular solution in contrast to caffeine contractures which can only be influenced after a prolonged perfusion with Ca-free saline (Zacharová and Uhrík 1970). These findings were explained on the assumption of coupling as well as activating role of extracellular calcium in the initiation of potassium contractures (Zacharová and Zachar 1967), and — based on biochemical investigations of microsomal fractions (Weber and Herz 1968; Carvalho 1968) — on the assumption of a direct effect of caffeine upon SR. In subsequent studies (Uhrík and Zacharová 1968, 1976) a marked swelling of SR cisternae in crayfish muscle fibres following activation by caffeine was described as distinguished from fibres undergoing potassium contracture, where only minor changes in SR ultrastructure could be detected.

The differences in the distribution of precipitates between caffeine- and potassium-induced contractures described in this work are compatible with the above-mentioned assumption. The pronounced decrease in intensity of SR precipitates and a moderate increase in density of myofibrillar precipitates after caffeine administration (Fig. 5) obviously reflects a shift of calcium from SR to the contractile apparatus; the activating calcium originates from the intracellular stores only. The direct effect of caffeine on SR vesicles of crab and crayfish muscle was

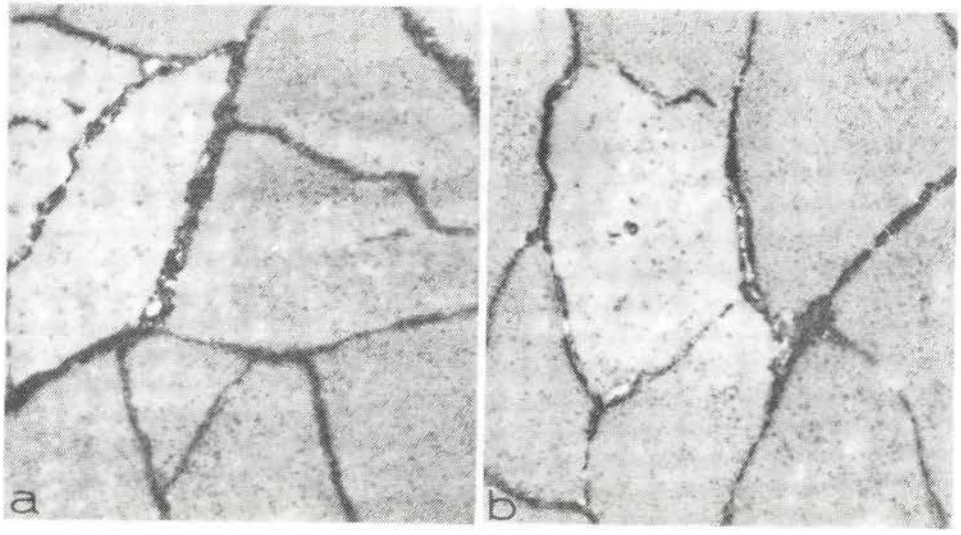

Fig. 9. Ultrathin sections. The fibre activated by tetanic stimulation (*b*) does not differ significantly from the control fibre (*a*). × 33,200.

proved also in biochemical studies using microsomal fractions (Huddart and Williams 1974; Tomková and Kontšeková 1980).

A markedly higher density of myofibrillar precipitates despite the only moderate decrease in density of SR precipitates during the potassium contracture (Fig. 8) suggests an involvement of extracellular calcium in a dual role of both coupling and activating agent. The participation of intracellular calcium in the contracture activation is difficult to assess quantitatively. The release of calcium from SR vesicles may be triggered by a mechanism of calcium-induced calcium release (for a review see Endo 1977).

Insignificant differences between the test and the control fibres during tetanic stimulation as opposed to the potassium depolarised fibres may be explained on the assumption that the potassium depolarisation encompasses the membranes of the clefts and the T-tubules in a more effective way, thus enhancing the overall Ca-influx. Whatever factor may be responsible, however, the insignificant changes after tetanic stimulation only stress the difference between depolarisation- and caffeine-induced activation.

The microscopic examination of differently activated fibres has also revealed that the sarcoplasmic reticulum of the crayfish behaved in a relatively uniform way: All SR elements along the sarcomere exhibited similar density of precipitates. This observation may be related to the relatively simple arrangement of SR in the crayfish (Brandt et al. 1965) exhibiting uniform shape of a fenestrated bag and lacking specialized configurations known in vertebrates.

Other internal or external Ca-stores (mitochondria, external lamina of sarcolemmal invaginations) do not seem to play a role as the sources of activating calcium in the crayfish muscle fibres. On the contrary, during different activation procedures, the density of mitochondrial precipitates increased, expecially during the potassium contracture. This would suggest a calcium-buffering function of mitochondria (for a review see Bygrave 1978) in the crayfish muscle. The dominating role of the SR as a calcium regulating structure in this type of crayfish muscle fibres is further stressed by the results of the stereological study (Uhrík et al. 1980): SR occupies 4.7 volume percent — markedly more than mitochondria (1.1 %), the T-system (1.1 %) or the sarcolemmal invaginations (0.7 %). If the total calcium content of the crayfish muscle fibre — estimated by Šajter et al. (1968) to be 10 mmol/l fibre — were sequestred within SR, its concentration would be about 200 mmol/l. This value agrees with that calculated for the barnacle giant fibre (Hoyle et al. 1973) where both the volume of the SR and the total calcium content are about ten times lower.

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