Effects of Ruthenium Red on Excitation and Contraction in Muscle Fibres with Ca²⁺ Electrogenesis

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Abstract. The effect of ruthenium red (RR) on the electrical and contractile responses, membrane Ca currents, staining patterns of the external and internal membrane system were tested in intact and mechanically skinned muscle fibres of the crayfish Astacus fluviatilis. The following results were obtained: 1. Depression of the contractile responses following membrane depolarization (twitch, tetanus, potassium contractures). 2. Caffeine contractures were unaffected in intact $(100 \,\mu \text{mol/l} - 1 \,\text{mmol/l} \,\text{RR})$ and blocked in skinned fibres $(30 \,\mu mol/l RR)$. 3. Mechanical threshold and mechanical latency were increased and/or prolonged. 4. The rate of depolarization of the action potentials (AP) was decreased and decremental spread of AP was recorded. 5. Both fast and slowly inactivating Ca ionic currents were decreased and the time constants of activation ($\tau_{\rm m}$) and inactivation ($\tau_{\rm h}$) were prolonged after RR (100 μ mol/l) pretreatment. 6. The penetration of RR into the T-system was inversely related to its binding to the sarcolemma. The depression of depolarization-induced contractions was most pronounced in fibres with unstained sarcolemma and stained T-tubules. In intact fibres, neither terminal cisternae nor other elements of SR were stained. On the contrary, all internal membrane structures were stained in skinned fibres. There was a gradient of staining intensity from surface toward the interior.

Key words: Ruthenium red — Crayfish muscle fibre — Electrical/Contractile activity — Ca²⁺ ionic currents — Ultrastructure

Introduction

Ruthenium red (RR) is a polycationic dye which stains extraneous coats of cells (Luft 1964, 1971 a, b) and has been used as a marker of anionic sites in muscle (Howse et al. 1970; Howell 1974; Frank et al. 1977; Forbes and Sperelakis 1979). The binding of RR to sarcolemma was shown to have an inhibitory effect upon Ca^{2+} binding (Madeira and Antunes-Madeira 1974) and could influence adversely the Ca^{2+} influx necessary for contractile activation (Kawamura and

Yabu 1978). In intact frog skeletal muscle fibres RR was shown to depress twitch tension, potassium contracture and Ca2+ influx (Suzuki et al. 1980) and to shift the contraction threshold in positive direction (Dorrscheidt-Käfer 1979). However, with purified RR, only a shift of action potential threshold and reduction in rates of depolarization, repolarization and conduction velocity were found, the effects on excitation-contraction (E-C) coupling being confirmed only for crude RR (Snowdowne and Howell 1984). In isolated vesicles of heavy fraction of sarcoplasmic reticulum (SR) (Ohnishi 1979; Miyamoto and Racker 1982) and in SR of skinned muscle fibres (Volpe et al. 1986) RR blocks Ca²⁺ permeability; this block is specific for calcium release channels of junctional SR (Fleischer et al. 1985; Smith et al. 1985). RR has thus become a tool for discriminating between high conductance Ca2+ release channels of junctional SR, identical with ryanodine receptors of junctional feet (Hymel et al. 1988; Lai et al. 1988), and other Ca^{2+} release pathways (for a review see Palade et al. 1989) including low conductance Ca²⁺ release channels (Smith et al. 1986; Suarez-Isla et al. 1986).

The dependence of crayfish muscle on extracellular calcium for both active electrical response and E-C coupling (Zacharová and Zachar 1967; Zachar 1981) makes the study of coupling mechanisms in this preparation interesting from comparative point of view. In our previous investigations (Uhrik and Zacharová 1982; Uhrík et al. 1985) 100 or 500 μ mol/l RR was used as a marker of negative surface charges in crayfish single muscle fibres. A conspicuous finding was an intense staining of peripheral segments of T-tubules, whereas the sarcolemma with its external lamina remained unstained. In a subsequent study (unpublished results) occasional staining of limited areas of crayfish sarcolemma could be detected. The binding of RR to the T-tubule membrane seems necessary for the inhibition of E-C coupling in frog muscles (Dorrscheidt-Käfer 1979; Suzuki et al. 1980). On the other hand, RR penetration into the T-system may be impeded by the repulsive forces of those RR^{6+} polycations already bound to sarcolemmal matrix (Snowdowne and Howell 1984). The RR binding pattern in the cravfish muscle offers an opportunity to relate staining of Ttubules to the inhibition of E-C coupling, and to test the assumption concerning the limited access of RR to T-tubules in areas of intense binding of the dye to sarcolemma.

In the present study the consequences of RR pretreatment on electrical and mechanical responses were examined in both intact and skinned crayfish skeletal muscle fibres. Preliminary results have already been published in an abstract form (Zacharová et al. 1989).

Materials and Methods

Experimental object

Experiments were performed on both intact and mechanically skinned, or internally perfused segments of muscle fibres dissected from m.extensor carpopoditi of the crayfish Astacus fluviatilis.

Experimental layout

After dissection in crayfish saline a single intact fibre was fixed in a measuring chamber to determine its length and diameter in two mutually perpendicular axes. The diameter was measured at least at 10 points along the entire length of the fibre. The fibre was then mounted in a perfusion chamber allowing rapid exchange of perfusion fluid (Zachar et al. 1964). To the distal tendon a hook was attached made from fine silver wire; it served to connect the single fibre to a silicon tensometer (Marko et al. 1986). The single fibres were stretched to one fifth of their slack length (l_0). Two platinum plate electrodes covered the lateral walls of the perfusion channel permitting the stimulation along the entire length of the fibre.

Recording of mechanical responses

Contractile responses (twitch, tetanus, potassium and caffeine contractures) were stored in a transient tester SE 561 (Austria) and then printed. The digitized samples were on-line fed into a SM4-20 computer. The software employed allowed the evaluation of amplitudes, areas, as well as the rapid and the slow activation and relaxation phase of the reponses.

Recording of electrical responses

Intracellular stimulation was performed and electrical membrane responses were recorded by means of microelectrodes. The recording microelectrodes were filled with 3 mol/l KCl (10–15 M Ω), the stimulating ones (3–5 M Ω) with 2 mol/l K-citrate. The pulse duration was 20–70 ms. The temperature of the solution in the experimental chamber was maintained at the required level by means of a thermistor-controlled cooling system.

The vaseline-gap voltage clamp method (Hille and Campbell 1976) was used to record Ca ionic currents in segments of single muscle fibres. The detailed experimental set-up has been described elsewhere (Zahradnik and Zachar 1987).

Ionic currents were recorded following analog compensation for the leakage and capacitance components and following filtering through a 10 kHz low-pass filter. The records were stored on magnetic medium using a PMD-85 microcomputer. The current traces were simultaneously photographed from the screen of a Tectronix S103N storage oscilloscope. The time courses of the slow and the fast Ca current component were determined from the total ionic current using an extended software (Pavelková et al. 1990), based on the equation of the Hodgkin—Huxley model for conductance (Hodgkin and Huxley 1952)

$$g_{\text{Ca}} = g'_{\text{Cat}} [1 - \exp(-t/\tau_{\text{mt}})]^6$$
, $\exp(-t/\tau_{\text{ht}}) + g'_{\text{Cas}} [1 - \exp(-t/\tau_{\text{ms}})^6$, $\exp(-t/\tau_{\text{hs}})$,

The above equation contains 6 parameters: three for the fast (f) and three for the slow (s) component.

The fitting ifself is relatively rapid (taking $1-2 \min$), also thanks to the sophisticated software which is highly flexible and allows stopping the fitting procedure at any point and resuming work with other parameters.

Solutions

Intact fibre. Van Harreveld solution contained (in mmol/l): Na⁺ 208.4, K⁺ 5.4, Ca²⁺ 13.5, Mg²⁺ 5.6, Cl⁻ 248.8; and Hepes 10 to keep pH at 7.3 - 7.5. Sr²⁺ solution was prepared by substituting 27 mmol/l SrCl₂ for 13.5 mmol/l CaCl₂ and 13.5 mmol/l NaCl in the crayfish saline.

Potassium contractures were mostly induced with solutions in which the external concentration of K ions $[K]_0$ was increased (8-fold or 32-fold), the product $[K]_0$. $[C]_0$ being kept constant. Propionate was substituted for chloride, and potassium for Na ions.

Caffeine in concentration of 6-10 mmol/l was directly dissolved in crayfish saline and used to elicit caffeine contractures.

Fibre segments. Single muscle fibres were cut and kept in internal solution which contained (in mmol/l): 240 Cs-glutamate or aspartate; 1.0 MgCl₂,; 0.01 Ca-glutamate; 5—10 EGTA; 0.2 cAMP. The pH was adjusted to 7.3—7.4. The extracellular solution perfusing the tested area of the segment contained (in mmol/l): 208.3 Cs-glutamate or aspartate; TEA/TMA glutamate respectively; and 13.5 Ca-glutamate.

Skinned fibres. Isolated single fibers were mechanically skinned in relaxation solution. The relaxing solution contained (mmol/l) K-glutamate 240, Mg-glutamate 1.0; Ca-glutamate 0.01; EGTA 5.0, ATP 5.0; cAMP 0.2; and Hepes to buffer the pH to 7.3-7.4. The fibre was transferred in this solution into the perfusion chamber. The shell segment of the fibre was fixed in the perfusion channel, the tendon segment was hooked onto a tensometer. Prior to the experiment, the fibre was streched by 1/5 of its slack length. Two other solutions were used: "jump solution" with the same composition as the relaxing one, except that EGTA was decreased to 0.1 mmol/l; and contracting solution, obtained by dissolving 6-10 mmol/l caffeine in jump solution. Ruthenium Red (BDH, England), $10 \mu \text{mol/l} - 1 \text{ mmol/l}$, was added into the experimental solutions from a stock solution in water.

Electron microscopy and microanalysis

Isolated fibres in the perfusion chamber were fixed for 30-45 min with 2% glutaraldehyde in 150 mmol/l sodium cacodylate buffer (pH 7.4), rinsed with the same buffer for 20 min, postfixed for 30 min with 1% OsO₄ in 150 mmol/l sodium cacodylate, and rinsed again for 5 min with the buffer. All fixative and rinsing solutions contained RR at concentrations coresponding to that used for physiological experiments (0.1 to 1 mmol/l). The specimens were then dehydrated in 70, 96 and 100% ethanol, cleared in propylene oxide and embedded in Durcupan.

Ultrathin sections were cut with a Porter-Blum MT2 ultramicrotome and examined in a JEOL JEM 1200/EX electron microscope at an accelerating voltage of 80 kV.

The presence of ruthenium in electron dense granules was detected by electron probe X-ray microanalysis with a LINK 860 X-ray spectrometer attached to the JEOL microscope. RuKa spectral line was used for ruthenium identification.

Results

Contractile responses

a) Twitch and tetanus. Fig. 1 illustrates the concentration and time dependence of RR action on single twitch and frequency tetanus responses elicited by



Fig. 1. Effect of ruthenium red on twitch (A) and tetanus (B) tension tested by massive stimulation. Ordinate: Peak tensions (%). Control tension of twitch (A) and tetanus (B) was taken for 100%. Abscissa: Duration of RR treatment at a: 10; b: 100; c: 500; and $d: 1000 \,\mu$ mol/l. Twitch and tetanus were elicited with supramaximal 2 ms pulses; (tetanus: 2 s at 50 Hz).

external stimulation along the entire fibre length. Low RR concentrations $(10 \,\mu\text{mo/l})$ had a weak effect. The inhibitory effect increased with the increasing concentrations of RR in the solution (100; 500; and 1000 μ mol/l). The twitch and the tetanus amplitude decreased approximately in parallel. At 100 μ mol/l RR, 50% of the initial twitch amplitude, as determined by regression analysis from the first, rapid decay phase, was reached after 26 min (n = 6). Upon raising RR concentration to 500 μ mol/l, the same was reached after 12 min (n = 3), and at 1000 μ mol/l after 7 min (n = 2). The time courses of tetanus decay are very similar (Fig. 1*B*).



Fig. 2. Inhibitory effects of RR (0.1 mmol/l) on potassium contractures. Single muscle fibres. K-contractures elicited at 43 mmol/l K⁺ in extracellular solution (A) and at 173 mmol/l (B) applied for 10 s. The first K-contracture: control solution; the second K-contracture after 25 minutes in RR (0.1 mmol/l); the last contracture 45 or 65 minutes respectively in control solution (vH) after washout of the RR solution.

b) Potassium contractures. Fig. 2 shows the blocking effect of RR (100 μ mol/l) on potassium contractures. Potassium contractures elicited by eightfold raising the external K⁺ concentration ($K_0 = 43 \text{ mmol/l}$) in the physiological crayfish solution were suppressed stronglier (A) than maximal contractures elicited by raising potassium concentration to 173 mmol/l. The amplitude and/or the area below the curve decreased to $12.4 \pm 3.5 \%$ (n = 4, p = 0.001) and $65 \pm 9 \%$ (n = 5, p = 0.02) in average for 43 and 173 mmol/l potassium contractures, respectively. Not only decreased the amplitude and area, but also the rate of the rapid phase contracture, reaching $57 \pm 6 \%$ (n = 5, p = 0.002). Only partial restoration was observed upon RR washout.

c) Caffeine contractures. RR suppressed membrane depolarization-evoked contractile activity, whereas it left caffeine contractures unaffected in this respect; the latters are known to be due to direct release of calcium ions from the sarcoplasmic reticulum. Fig. 3 compares RR-induced changes of electrical stimulation-elicited contractile activity (twitch and tetanus) with those elicited by washing the same fibre with caffeine solution. Obviously, even a high RR concentration (1 mmol/l) did not suppress caffeine contracture, whereas both twitch and tetanus were considerably suppressed even after short-term RR action. A similar pattern was observed also in four other fibres, even at lower



Fig. 3. The effect of RR on caffeine contractures. *A*: Control responses: twitch, tetanus and caffeine contractures (6 mmol/l) respectively. *B*: Mechanical responses 5 min (twitch), 10 min (tetanus) or 35 min (caffeine contracture) in RR (1 mmol/l) solution.

RR concentrations (100 and 500 μ mol/l). Caffeine contractures showed slight facilitation, with their area increasing to 124.5 \pm 8 %.

Simultaneous recording of electrical and contractile activity

Since RR suppressed extracellular stimulation-elicited contractile activity, a further series of experiments was designed to investigate the correlation between electrical and contractile activity of muscle fibres under RR action.

Electrical and contractile activity in response to intracellular stimulation were recorded in two media: normal physiological saline (Fig. 4*A*), and a solution with Sr ions substituted for Ca ions (Fig. 4*B* and *C*). In the presence of Sr ions (27 mmol/l) the local gradual response turns to action potential spreading along the fibre. Fig. 4*B* and *C* show two strontium action potentials. The first one was recorded with the stimulating and the recording electrodes being approx. 500 μ m from each other (*B*), the other one at a distance of



Fig. 4. Changes of electrical and contractile responses in RR. *A*: Records from a single muscle fibre immersed in van Harreveld (vH) solution ($Ca^{2+} = 13.5 \text{ mmol/l}$) (first record) and after 5; 23; and 30 minutes respectively in vH solution containing 0.1 mmol/l RR. *B* and *C*: Action potentials and twitches in strontium solution (Ca^{2+} replaced with 27 mmol/l Sr²⁺). The distance between the recording and the stimulating microelectrode was approx. 500 μ m (*B*) or 1500 μ m (*C*). Records in RR were obtained 9 and 17 minutes after RR pretreatment. The bottom traces are controls (30 and 9 min respectively) after washout of the experimental solution.

1500 μ m between the electrodes (C). The addition of RR (100 μ mol/l) into the respective solution induced gradual changes in electrical and contractile responses. The contraction amplitude decay set on earlier and was more marked than amplitude changes of the local electrical response or those of action potential. However, the amplitude of action potential recorded from the more distant site as well as the depolarization phase rates of both AP decreased. Tables 1 and 2 summarize changes of electrical and contraction parameters recorded after 25 —30 min of RR action in normal physiological saline (Table 1) and upon

Parameters	van Harreveld solution (vH)	vH + RR 100 µmol/l after 25—30 min treatment	vH after 25-30 min rewashing 71.8 ± 0.5 (n = 9) 31.9 ± 1.9 (n = 6)	
Resting potential (mV)	72.15 ± 1.2 (<i>n</i> = 13)	72.6 ± 1.8 (<i>n</i> = 10)		
Local electric responses amplitude (mV)	30.2 ± 2.0 (<i>n</i> = 6)	$*36.4 \pm 1.7$ (<i>n</i> = 6)		
Rate of maximal depolarization dV/dt (mV/ms)	0.64 ± 0.06 0.85 ± 0.1		0.68 ± 0.07	
Mechanical threshold (mV)	22.09 ± 3.3 (<i>n</i> = 11)	$*30.18 \pm 1.46$ (<i>n</i> = 11)	28.56 ± 1.2 (<i>n</i> = 9)	
Mechanical latency (ms)	27.8 ± 2.5 (<i>n</i> = 9)	*33.3 ± 3.9 (n = 9)	34.4 ± 3.6 (<i>n</i> = 9)	
Mechanical response amplitude (%)	100	$*62.3 \pm 12$ (<i>n</i> = 6)	53.6 ± 15 (<i>n</i> = 6)	
Shortening rate (mg/ms)	0.47 ± 0.1 (<i>n</i> = 7)	$*0.21 \pm 0.05$ (<i>n</i> = 7)	0.32 ± 0.01 (<i>n</i> = 6)	
Half amplitude relaxation rate (mg/ms)	0.14 ± 0.03 (<i>n</i> = 7)	$*0.042 \pm 0.009$ (<i>n</i> = 7)	0.055 ± 0.014 (<i>n</i> = 6)	

Table 1. Effect of Ruthenium red on local electrical and contraction parameters

Notes: Means \pm SEM are shown; n – number of single muscle fibres; * significance at P = 0.05

substituting Sr ions for Ca ions (Table 2). All the parameters studied undergo qualitatively similar changes (raised mechanical threshold, prolonged mechanical latency, decreased rates and amplitudes of both the activation and the relaxation phase of muscle contraction). Different changes concerned the amplitude and rate of the local electrical response activation phase and of the action potential. The local electrical response increased from $30.2 \pm 2 \text{ mV}$ to $36.4 \pm 1.7 \text{ mV}$, and the action potential depolarization phase rate decreased significantly, and the decremental spread of action potential was more marked.

Parameters	van Harreveld Sr solution (vH Sr)	vH Sr + RR 100 µmol/I (30 min)	vH Sr (20—30 min)	
Resting potential (mV)	66.2 ± 0.8 (<i>n</i> = 9)	64.0 ± 1.07 (<i>n</i> = 16)	68.0 ± 1.9 (<i>n</i> = 8)	
AP (mV) 500 µm	68 25 + 3 7	63.62 + 1.8	66.7 + 1.46	
1500 µm	67.5 ± 1.45	$*54.25 \pm 2.4$	63.9 ± 1.8	
1500 μ	(n=4)	(n = 4)	(n=4)	
AP duration (ms) 500 µm	22.0 ± 1.1	24.8 ± 4.8	22.7 ± 0.9	
1500 µm	20.5 ± 1.3	22.0 ± 5.3	24.0 ± 2.1	
	(<i>n</i> = 4)	(<i>n</i> = 4)	(<i>n</i> = 4)	
AP depolarization rate 500 µm	4.1 ± 0.38	*2.8 ± 0.45		
(mV/ms) 1500 µm	3.43 ± 0.44	*2.03 ± 0.27		
	(<i>n</i> = 4)	(<i>n</i> = 4)		
Mechanical	18.3 ± 3.1	*33.4 ± 2.5	23.2 ± 2.6	
latency (ms)	(n = 8)	(n = 8)	(n = 8)	
Twitch amplitude (%)	100	*18 ± 2	52 ± 13	
	(<i>n</i> = 4)	(n = 4)	(n = 4)	
Shortening rate	2.16 ± 0.2	*0.63 ± 0.2	0.15 ± 0.1	
(mg/ms)	(<i>n</i> = 4)	(n = 4)	(n = 4)	
Half amplitude relaxation	0.76 ± 0.2	*0.16 ± 0.08	0.31 ± 0.06	
rate (mg/ms)	(n = 4)	(n = 4)	(n = 4)	

Table 2. Effects of RR on action potential and contraction

Notes: Means \pm SEM are shown; *n* — number of single muscle fibres; * significance at *P* = 0.05 500 and 1 500 μ m — distances between the stimulating and recording microelectrodes

Ca ionic currents

Ca ionic currents recorded in perfused segments of crayfish muscle fibres are of two-component nature (Zahradnik and Zachar 1982). A detailed analysis showed that there are two types of Ca channels (Zahradnik and Zachar 1987; Jdaïâa and Guilbault 1986). One of them, mostly arising at lower depolarizations, inactivates rapidly, whereas the other Ca channel type inactivates slowly. Fig. 5 illustrates the effect of RR on Ca ionic currents recorded in 4 segments at low membrane depolarization (MP -40 and -30 mV), at which the rapidly inactivating current is more or less isolated. Regardless of the replacements of Na⁺



Fig. 5. The effect of R R (0.1 mmol/l) on total calcium ionic currents. Extracellular solution superfusing the tested area of the segment contained (in mmol/l): *a*) Ca^{2+} 13.5 and Cs^+ glutamate 240; *b*) Ca^{2+} 13.5 and Cs^+ aspartate 240; *c*) TEA⁺ glutamate 240 and Ca^{2+} 13.5; *d*) TMA⁺ glutamate 240 and Ca^{2+} 13.5, 1: Control records, 2: Test records, 3: Recovery. Four different segments from four isolated muscle fibres.

and Cl⁻ ions in the solution washing the tested fibre segment (Cs⁺, TEA⁺, TMA⁺, glutamate, aspartate), 100 μ mol/l RR suppressed, as soon as within the first 2—5 minutes, predominantly the inactivating current. After RR washout, restoration could be recorded only exceptionally, as a rule the amplitude decrease continued. If RR was allowed to act only for a short period of time (2 min, Fig. 5*C*), an increase of ionic current could be recorded after rapid RR washout. The conductance of Ca channels decreased and the time constants of activation (τ_m) and inactivation (τ_h) of both Ca currents were prolonged (Table 3).

Skinned fibres

In previous experiments (see mechanical responses) we could show that RR facilitated rather than suppressed caffeine contractures elicited in intact muscle fibres. However, experiments on vesicles isolated from the SR of vertebrate muscle suggested that RR inhibits both the Ca release channels localized in the SR terminal cisternae (Miyamoto and Racker 1982; Smith et al. 1985) and caffeine induced Ca efflux (Palade 1987). Mechanical responses to change from relaxing solution (5 mmol/l EGTA) to contracting solution (0.1 mmol/l EGTA)

Ca current	Membrane depolariza- tion (mV)	Amplitude	GmS/cm ²	τ _m (ms)	$ au_{h}$ (ms)
Inactivating [-30] [-40 tc +10]	[-30]	$58.6 \pm 8.4\%$ (<i>n</i> = 7)	0.65 ± 0.05 (<i>n</i> = 7)	1.19 ± 0.07 (<i>n</i> = 7)	1.13 ± 0.13 (<i>n</i> = 7)
	[-40 to +10]		0.69 ± 0.06 (n = 21)	1.12 ± 0.05 (n = 20)	$\$1.16 \pm 0.12$ (n = 21)
Non-inacti- vating	[-30]	$71.03 \pm 8.8\%$ (<i>n</i> = 7)	0.879 ± 0.109 (<i>n</i> = 7)	1.22 ± 0.06 (<i>n</i> = 7)	$\$1.05 \pm 0.07$ (<i>n</i> = 7)
	[-40 to +10]		0.82 ± 0.05 (<i>n</i> = 19)	1.13 ± 0.05 (<i>n</i> = 19)	1.1 ± 0.045 (n = 19)

Table 3. Effects of RR on Ca ionic curents and calcium conductance parameters

Notes: Means \pm SEM are shown; n — number of single muscle fibres;

all values except those denoted by § are significant at P = 0.05.

and 10 mmol/l caffeine) were studied in 12 skinned fibres. Rapidly developing responses were observed in 8 fibres which relaxed upon washing with the relaxing solution (Fig. 6). If the relaxing solution was allowed to act for a sufficiently long interval (60-70 min), further caffeine contractures could be evoked (Fig. 6A). The amplitude of the second caffeine contracture was approximately identical (as was the case in the experiment shown) or lower than that of the preceding one, reaching 71.75 + 10.97% of the preceding contracture height (4 fibres). If however 20 min prior to the second contracture the fibre was washed with a solution containing 30 µml/l RR, the second caffeine contracture activated with a delay, and with a slowlier rate than the first one. The rapid phase of the caffeine contracture was suppressed (Fig. 6B, C). At the same interval after caffeine application, the amplitude was only 25.1 + 13.5% of that of the preceding contracture (n = 5, p < 0.01). Following RR washout, the rapid phase of the caffeine contracture restituted but partially (Fig. 6B) or remained suppressed (Fig. 6C). The caffeine contracture amplitude reached only 27.25 ± 0.6 % of that of the control contracture.

Only low and slowly activating contractures could be recorded in 4 skinned fibres following caffeine application, which relaxed upon placing the fibre into the relaxing solution. No effect of RR $(30 \,\mu \text{mol/l})$ on these contractures was observed (2 fibres).



Fig. 6. Caffeine contractures in skinned crayfish muscle fibres. Inhibition after RR pretreatment. A: Repeated caffeine contractures elicited in the same skinned fibre. Skinned fibre soaked in "relaxing" solution (5 mmol/l EGTA) was perfused with "jump solution" (0.1 mmol/l EGTA) for 3—4 min and then with "contracting" solution (0.1 mmol/l EGTA and 8 mmol/l caffeine). The relaxing solution was used for washout. The second caffeine contracture was elicited 70 min after the first one using the same protocol. The third contracture was recorded after 18 hours; during this period the skinned fibre was kept in the relaxing solution in a refrigerator. B and C: Two different skinned fibres. The first column shows control caffeine contractures; the subsequent contractures were recorded after 20 minutes in 30 μ mol/l RR. The third (control) contractures were elicited 60 minutes after washout of the RR solution.

Electron microscopy

a) Intact fibres. Following electrical stimulation before and after RR applica-



Fig. 7. Different RR staining patterns in crayfish muscle fibres. e — external lamina. Magnification: \times 5100 (*A*), \times 8200 (*B*), \times 6200 (*C*), \times 11,200 (*D*).

tion (0.1; 0.5; or 1 mmol/l RR for 20 to 90 min) 6 fibres were processed for ultrastructural examination. Different staining patterns were encountered in individual fibres as well as in different segments of the same fibre: (*a*) Unstained sarcolemma (plasma membrane with external lamina) with extensive staining of T-tubules in the peripheral layer of the fibre (Fig. 7*a*); (*b*) faint staining of the



Fig. 8. X-ray spectrum of electron dense structures after RR pretreatment. A prominent RuK α peak is present in addition to osmium peaks (due to fixative solution containing OsO₄).

sarcolemma with a moderate to extensive staining of T-tubules (Fig. 7*b*); (*c*) heavy staining of the marginal thin layer of the external lamina with no T-tubule staining (Fig. 7*c*); (*d*) heavy staining of the whole sarcolemma with only short stretches of stained T-tubules (Fig. 7*d*).

In fibres with prevailing (a) and (b) type of staining the depression of the contractile response was pronounced. In fibres with predominant (c) or (d) staining pattern the contractile activity was lowered only insignificantly or not at all, in one case the contractions were even higher than before RR application.

The presence of ruthenium in electron dense deposits was verified by electron probe X-ray microanalysis (Fig. 8). RR did not penetrate in visually or analytically detectable quantities into the sarcoplasm. Where dyads were found only membranes of the T-tubules were electron dense, the terminal cisternae of the sarcoplasmic reticulum remained unstained (Fig. 9*A*).

b) Skinned fibres. The surface of skinned fibres was covered with mitochondria or SR vesicles surrounding adjacent myofibrils (Fig. 9B). The mitochondria or SR vesicles located at the boundary between intra- and extracellular spaces were stained heavily by RR. The intensity of staining inside the fibre decreased, nevertheless RR was always present in form of tiny granules delineating the vesicular membranes (Fig. 9C).

In places, small patches of the plasma membrane remained attached to the fibre surface (Fig. 9D). Typically, such patches were stained intensely by RR (*arrowheads* in Fig. 9D). Mitochondria located beneath these patches were almost unstained as opposed to heavily stained denuded mitochondria in the immediate vicinity (*arrows* in Fig. 9D).

It was not possible to distinguish T-tubules from SR after the skinning



Fig. 9. *A*: A dyad inside a crayfish muscle fibre after RR pretreatment. Only the T-tubule is stained. *B*, *C*, *D*: RR staining in skinned muscle fibres. Magnification: \times 78400 (*A*), \times 8300 (*B*), \times 16800 (*C*), \times 6300 (*D*).

procedure. The interfibrillar space was occupied by swollen vesicles, the dyads could not be unambiguously identified.

Discussion

The effects of RR on electrical and mechanical responses of crayfish muscle fibres (the electrical responses of which are dependent on extracellular calcium, Fatt and Ginsborg 1958; and the contractile responses on tubular Ca^{2+} , Zacharová and Zachar 1967; Valko et al. 1967; Lacinová and Poledna 1990) resemble in several parameters those described for frog muscle fibres (Suzuki et al. 1980; Dorrscheidt-Käfer 1979), although the former operate on the sodium electrogenesis principle and are less dependent on extracellular Ca^{2+} .

Similar changes concerned contractile responses (twitch, tetanus and potassium contractures) which were suppressed, and caffeine contractures which remained effectively unchanged even at high RR concentrations. Also, the mechanical threshold increased, and the mechanical latency was prolonged correspondingly.

Since in frog muscle fibres, membrane and action potentials as well as their overshoots showed only little change, the RR effect has been attributed to changes occurring within the excitation-contraction coupling cascade (Suzuki et al. 1980). This conclusion was questioned after the observation that low concentrations $(3-10 \,\mu \text{mol/l})$ of purified RR facilitated twitches, while leaving the shift of the mechanical threshold towards positive depolarization unaffected (Snowdowne and Howell 1984); moreover, RR as a polycationic substance does not induce changes in E—C coupling similar to those induced by Ca²⁺ (Howell and Oetliker 1987). The authors have considered these results functional evidence to support the morphological observations that RR does not penetrate into the tubular system of frog muscle (Howell 1974).

Recent works using mammalian muscle (Delbono and Kotsias 1989) and heart muscle (Gupta et al. 1988) have shown that low concentrations (5— $10 \,\mu$ mol/l) of even unpurified RR facilitate twitches and that the drug has biphasic, inhibitory and facilitating, effects in dependence on the concentration used (Gupta et al. 1988).

In our experiments, we also could occasionally observe twitch facilitation or a shift of the current-voltage characteristics of Ca ionic currents towards negative potentials at low concentrations ($10 \mu mol/l$) or within the first minutes following the application of even high RR concentrations ($100 \mu mol/l$) (not shown).

For crayfish muscle fibres, the effective inhibitory RR concentration was higher (100 μ mol/l) than reported for the frog muscle. This may be connected to the higher physiological concentrations of Ca ions in the crayfish hemolymph as compared to the frog, and/or to the use of unpurified RR in our experiments. Some observations have suggested that larger Ca concentrations require larger

RR concentrations to produce the same effect (Dorrscheidt-Käfer 1979; Person and Kuhn 1979; Kanmura et al. 1989).

The technique of the simultaneous recording of electrical and contractile responses allowed us to correlate changes developing in both parameters. We could not find reports of simultaneous recordings of both responses obtained from other experimental objects. Data summarized in Tables 1 and 2 show that RR leaves the resting membrane potential unaffected, while decreasing the rate of the action potential depolarization phase; this is then manifested in an increased mechanical threshold and in a prolongation of mechanical latency. RR accentuates the decrementing spread of action potential along the fibre.

A major finding however concerned the blocking effect of RR on Ca ionic currents recorded in internally perfused segments of crayfish muscle fibres upon substituting less permeable Cs ions or other ions (TEA⁺, TMA⁺) that suppress K conductance, for K⁺ and Na⁺ ions. Data shown in Table 3 demonstrate that RR suppressed both types of Ca ionic currents, the fast inactivating Ca ionic current and the slowly inactivating Ca current. Similarly as from mammalian muscles (Curtis and Catteral 1984; Glossman et al. 1987; K rižanová et al. 1988), Ca channel could be also isolated and purified from the tubular fraction of crayfish muscle (Križanová et al. 1990). This channel was incorporated into planar lipid bilayers, and a conductivity of 16 pS could be measured (Hurňák et al. 1990). So far, the in vitro sensitivity of the channel to RR has not been tested.

RR (1 mmol/l) has been observed to decrease Ca⁴⁵ influx into smooth muscle (Greenberg et al. 1973) and frog muscle upon potassium-induced depolarization (Suzuki et al. 1980). Baux et al. (1979) could not observe any effect of 20 μ mol/l RR on Ca²⁺ spike of Aplysia neurons. Stimers and Byerly (1982) however could observe inhibition of Ca ionic currents in perfused snail neurons by 100 μ mol/l RR, which agrees with our own observations. According to these authors however, the action of 100 μ mol/l RR is nonspecific as it suppressed also voltage-dependent K channels. On the contrary, RR is highly specific for Na channels since delayed inactivation of Na currents by RR can be observed already at nanomolar concentrations.

The enhanced local electrical response observed under RR action and stimulation in normal physiological saline might be explained assuming that RR affects K^+ conductance also in crayfish muscle fibres. The muscle membrane of our experimental species investigated contains three types of potassium conductance and/or potassium channels (Henček et al. 1978). The rapid K channel (Mounier and Vassort 1975; Henček et al. 1978) activates shortly after the Ca conductance activation, thereby preventing the generation of calcium action potential. Consequently, suppression of the rapid K channel results in an enhancement of the local response Ca component. The decrease of the delayed K conductance results in a widening of the action potential. Widening of frog

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muscle fibre action potential has been recorded at low RR concentrations by Snowdowne and Howell (1984), and by Delbono and Kotsias (1989) who worked with mammalian muscle. This may explain also the facilitation effect on muscle contraction recorded in their experiments. RR-induced changes of electrical parameters, including those concerning ionic currents, suggest that RR affects both the contraction and the ionic mechanism of calcium action potential. Different sensitivities of the individual ionic channels to RR may then be reflected in the resulting electrical as well as contraction changes.

Another remarkable observation has been that following RR washout (25—30 min), the parameters of electrical responses (local electrical response, action potential amplitude, rate of depolarization and decremental spread) are restored to their respective initial values sooner than are contraction parameters, with a resulting partial dissociation of E—C coupling. The reversible changes might be attributed to the reversible RR binding with negatively charged groups of the membrane surface, and partly to irreversible binding with charged groups fixed at the surface of the T-tubules walls, similarly as proposed for frog muscle (Dorrscheidt-Käfer 1979).

The partial irreversibility of contractile responses shows a good correlation with the poor reversibility of Ca ionic currents following RR washout. This may be explained by inactivation of Ca channels present in the muscle fibre tubular system (Zahradnik et al. 1984; Križanová et al. 1990; Hurňák et al. 1990).

Assuming that the Ca dihydropyridine receptor represents a sensor for Ca release channel activation also in crayfish muscle fibres (Križanová et al. 1990), similarly as postulated for vertebrate muscle (Rios and Brum 1987), and that there is a direct interaction between molecular components of the transverse tubule and those of the sarcoplasmic reticulum (Block et al. 1988; Wagenknecht et al. 1989), RR could partially paralyze E—C coupling. This assumption has real foundations, since all the principal components of E—C coupling have recently been described in crayfish muscle fibre (Zachar and Zacharová 1989; Formelová et al. 1990; Hurňák et al. 1990); moreover, their biophysical, ultra-structural and biochemical characteristics are very similar to those of vertebrate muscle.

RR binding to muscle membranes is another determining factor. Since membrane staining is observed already at functionally effective concentrations (100 μ mol/l and higher), we could investigate the relation between staining and changes of contractile responses.

Whereas in other cell types, including heart and vertebrate skeletal muscles, external coats are the preferential site for RR binding, the situation in crayfish muscle is unique in that different staining patterns may be observed even along the same fibre.

The relation between the staining patterns and depolarization-induced contractions has shown relatively small changes in fibres with heavily staining sarcolemma (Figs. 7c and 7d). On the other hand pronounced inhibition of contractions was observed in cases with little or no RR binding to the external lamina, and with stained T-tubules (Figs. 7a and 7b). Thus, the inhibition of mechanical responses following membrane depolarization (twitch, tetanus, Kcontracture) seems to be related to the ability of the dye to enter the T-tubule lumen and to bind to its membranes. The binding of RR to the external lamina is critical for the outcome of this process. Since fibres with inhibited contraction showed normal or even enhanced caffeine contractures it may be assumed that the penetration of RR into the T-tubules rather than into the sarcoplasm was responsible for the inhibitory effect. RR entering the SR of intact fibres has been described only after prolonged (overnight) exposure of muscles to RR solutions, the procedure having deleterious effects on the majority of fibres (Howell 1974). Apparently, the plasma membrane in cravfish, even if present as small patches in skinned fibres is a hardly permeable barrier to RR diffusion.

The form and extent of RR binding to the sarcolemma are of importance for the dye penetration into the T-system tubules and this gives support to the idea of self-imposed diffusion barrier of already bound RR⁶⁺ polycations (Handley and Chien 1981; Snowdowne and Howell 1984). The differences in sarcolemmal RR staining patterns occurring even along the periphery of the same crayfish single muscle fibre are difficult to explain; they might reflect different composition of the matrix constituting the external coat in this animal species in comparison with vertebrate muscles.

The skinning procedure yielded preparations with the inner sarcoplasmic space accessible to RR as seen by fine granular staining of all membrane components. The intensity of this staining, however, decreased markedly from the periphery toward the interior of fibres.

As distinct from intact fibres, caffeine contractures in skinned fibres were depressed by RR. This is in keeping with the blocking effect of RR on caffeine induced Ca^{2+} efflux in isolated heavy vesicles of SR (Kirino and Shimizu 1982; Palade 1987), the receptor for caffeine being located in Ca^{2+} release channels of the same fraction (Rubtsov and Murphy 1988).

An interesting finding in our study concerns a specific inhibitory effect of RR on the first fast phase of the caffeine induced contracture in skinned fibres, the second slow phase being more or less unaffected (Fig. 6). The oscillatory pattern of caffeine contracture elicited in intact crayfish muscle fibres by lower caffeine (6 mmol/l) has been described previously (Zacharová et al. 1968; Uhrík and Zacharová 1968; 1976) and interpreted in terms of a gradual recruitment of different calcium stores. Recent data concerning the presence of low conductance Ca^{2+} release channels in SR vesicles from vertebrate muscles and their

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activation by caffeine (Suarez-Isla et al. 1986; Irribarra et al. 1988) suggest that the second peak of caffeine contracture may result from opening of RR insensitive low conductance Ca^{2+} release channels.

Ca release channels (ryanodine receptors) have recently been described in sarcoplasmic reticulum of the crayfish (Formelová et al. 1990). Ca release channels incorporated into planar lipid bilayers show two conductance states (Hurňák et al. 1990), and two populations of Ca release channels have been reported also in native SR of the crayfish muscle (Poláková et al. 1990).

A more comprehensive understanding of caffeine induced mechanical activity in crayfish muscle could be derived from the study of the pharmacological characteristics of these channels.

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