Modulation by Adrenaline of Electrophysiological Membrane Parameters and Contractility in Intact and Internally Perfused Single Muscle Fibres of the Crayfish

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Abstract. (1) The effect of catecholamines on basic membrane characteristics (including labeled ionic fluxes) and contractile parameters was followed in current clamp and voltage clamp conditions in intact muscle fibres and internally perfused muscle fibre segments respectively of the crayfish *Astacus fluviatilis*; i.e. in muscle fibres which spike and activate tension on calcium principle. (2) Both adrenaline and noradrenaline (6.10^{-6} mol/l) facilitated twitch tension induced by graded membrane responses or strontium all-or-none spikes. No effect of isoprenaline was observed. (3) Adrenaline (6.10^{-6} – 6.10^{-5} mol/l) produced an inotropic effect, which appeared with a latency of 2 min and reached its maximum in 5 min. The rates of activation and relaxation of contraction were increased, whilst the latency and the threshold depolarization were decreased. The changes persisted (several tens of min) after washout of adrenaline, depending on concentration and duration of adrenaline application. (4) The resting potential and the strontium spike (Ca^{2+} replaced with Sr^{2+}) were not influenced and the graded responses were facilitated by adrenaline (from 36.4 ± 1 mV to 40.0 ± 2 mV; RP = 77.2 ± 0.5 mV). (5) Extracellular Ca^{2+} ions are required for the inotropic effect of adrenaline to occur. The decrease of electrical and contractile responses in nominal calcium-free solutions or after a blockade of Ca^{2+} influx by Ni^{2+} ions (1 mmol/l) was relieved by adrenaline. The persistence of inotropic effect of adrenaline was absent, when the extracellular concentration of Ca^{2+} ions, $[Ca^{2+}]_0$ was decreased from 13.5 to 3.4 mmol/l or the Ni^{2+} ions were added. The influx of $^{89}Sr^{2+}$ ions was decreased in the presence of Ni^{2+} ions from 24.2 ± 4.7 pmol.cm^{-2}.s^{-1} to 11.0 ± 2.8 pmol.cm^{-2}.s^{-1}, but restored to 20.4 ± 5.8 pmol.cm^{-2}.s^{-1} in the presence of adrenaline (6 μmol/l). (6) Adrenaline itself decreased the influx of $^{89}Sr^{2+}$ ions, and prolonged the time

constant of efflux both in resting and stimulated fibres. (7) The effect of adrenaline is dependent on mobilization of Ca^{2+} ions from the sarcoplasmic reticulum. First, the inotropic effect of adrenaline was absent in the presence of procaine (blockator of the Ca release channel of the SR), in spite of the increase of the active membrane response (all-or-none procaine action potential); second, adrenaline accelerated the uptake of Ca ions by SR as evidenced by shortening of the restitution processes after caffeine contractures by adrenaline. (8) Membrane calcium currents are increased by adrenaline as a rule; mainly at lower depolarizations (−50 to −20 mV). All components of the whole calcium conductance, which differ by time constants of activation τ_m and inactivation τ_h (i.e. of the fast, the intermediate and the slow calcium conductance respectively) are increased in the presence of adrenaline. The calcium conductance of the fast component was increased by $31 \pm 6.3\%$, of the slow component by $132.2 \pm 27.6\%$ and of the intermediate component by $101.5 \pm 20.9\%$ ($n = 22$, $P = 0.01$–0.001). The enhancement of calcium currents persisted after the withdrawal of adrenaline from the bathing solution. The time constants of activation, τ_m were not significantly changed; the time constant of inactivation, τ_h in the fast calcium channel was prolonged.

Key words: Adrenaline — Noradrenaline — Inotropic effect — Calcium currents — Calcium conductances — Labelled fluxes — Graded electrogenesis — Strontium action potential — Procaine spike — Ni^{2+} ions — Contraction — Caffeine contractures — Skeletal muscle fibre — Muscle fibre segments — Crayfish muscle — Calcium dependent electrogenesis — Internal perfusion — Voltage clamp

Introduction

It has been known for a long time, that catecholamines influence contractility in skeletal muscles in dependence on the type and the functional state of the muscle and the experimental object (for a review see Bowman and Nott 1969, Bowman 1981, Williams and Barnes 1989b). The disputable question, if adrenaline has a positive inotropic effect on amphibian skeletal muscle was answered, when Oata and Nagai (1977) and mainly Gonzales-Serratos et al. (1981) analyzed the mechanism of inotropic effect of adrenaline on intact and skinned muscle fibres of the frog. Their results provide evidence that adrenaline stimulates through cyclic AMP the Ca pump of the sarcoplasmic reticulum (SR) increasing thereby the concentration of Ca^{2+} within the SR and this extra Ca^{2+} released during subsequent activation may produce the increase in twitch tension.

Agents that increase intracellular cAMP level increase Ca^{45} influx in cultures of skeletal muscle cells from chick embryos (Schmid et al. 1985). Activation of purified skeletal muscle Ca channels incorporated into planar lipid bilayers by cAMP dependent proteinkinase was documented by Flockerzi et al. (1986).

Adrenaline and cAMP increase the amplitude of both the slow and fast calcium currents in frog muscle fibres (Arreola et al. 1987) and the same changes were even observed after application of phenylephrine, an α-adrenergic activator (Stefani et al. 1987).

The importance of extracellular Ca^{2+} ions for the inotropic effect of adrenaline was corroborated by Williams and Barnes (1989a) in twitch and by Huerta et al. (1991) in tonic frog muscle fibres respectively.

In skeletal muscle G-proteins modulate the activity of Ca channels either by acting directly on the channel or through a cAMP- dependent phosphorylating mechanism (Yatani et al. 1988). Activation of G-proteins by GTPγS can induce force generation in skinned skeletal muscle (Di Virgilio et al. 1986; Villaz et al. 1989) as well as an increase in both charge movement and Ca current (Garcia et al. 1990). It should be mentioned, however, that Lamb and Stephenson (1991) did not observe changes in charge movement. GTPγS can cause a calcium- induced Ca release as a result of enhanced calcium entry through the DHP sensitive calcium channels (Somasundaram 1991). Stimulation of calcium slow channels in bullfrog skeletal muscle fibre both by cAMP and cGMP was observed quite recently (Kokate et al. 1993).

In heart muscle the increase of permeability for Ca^{2+} ions to adrenaline was determined by means of labeled ^{45}Ca fluxes (Reuter 1965) as well as by electrophysiological techniques (for review see Reuter 1979, Reuter 1987). As follows from the voltage- and patch-clamp measurements, the increased influx of Ca^{2+} into the myocyte due to adrenaline and other β-ergic agonists is realized via the L-type calcium channels. β-adrenergic stimulation of calcium current (I_{Ca}) in heart cells is due to Ca channel phosphorylation by enhanced cAMP-dependent proteinkinase A activity (for review see Hartzell 1988, Peltzer et al. 1990, Trautwein and Hescheler 1990). Phosphorylation of the Ca channel increases the probability of the channel being in the open state by potentiation of high activity gating modes (Yue et al. 1990). Contradictory results are, however, reported concerning the influence of the β-adrenergic drugs on T-type Ca channel (Bean 1985, Mitra and Morad 1986, Tytgat et al. 1988, Tseng and Boyden 1989, 1991, Tytgat et al. 1990, Alvarez and Vassort 1992). There are some indications that the α receptors could influence the T-type Ca channel (for a review see Terzic et al. 1993).

From comparative physiological view an interesting question concerns the mechanism of action of adrenaline in skeletal muscle fibres with a different type of excitation-contraction coupling mechanism as assumed to be present in skeletal muscle fibres of vertebrates (for a review see Rios et al. 1991).

In our experimental approach we used single skeletal muscle fibres of the crayfish, where the active membrane responses and contractility are dependent on the extracellular Ca ions and probably the E-C coupling mechanism is similar as in car-

diac muscle (for a review see Zachar 1971, Suarez Kurtz 1982, Palade and Gyorke 1993)

The effect of bioamines (5-hydroxytryptamine, serotonin, octopamine, dopamine) on contractility in crustacea was studied in several studies They act predominantly via facilitation of processes in synaptic transmission as well as by direct action on muscle fibre structures (for review see Kravitz et al 1980, Kravitz 1988) Positive inotropic effect of adrenaline on nerve evoked contraction and potassium contracture was found by Huddart and Battram (1984) in the claw dactyl closer muscle of the crab

The fact that the octopamine, often considered as counterpart of noradrenaline, potentiates in the opener muscle of the crayfish, caffeine induced tension to the same extent as it increases tension resulting from depolarization, lead Fisher and Florey (1987) to postulate that octopamine could enhance not only the Ca entry (as suggested by Kravitz et al 1980), but also the Ca release

Influx of Ca ions into the muscle cell of vertebrates, as we mentioned above in connection with skeletal and heart muscle, could also take place in vertebrate skeletal muscle via different calcium channels The existence of two calcium channels was demonstrated to exist in internally perfused muscle fibre segments by Zahradnik and Zachar (1982, 1987) Two Ca channels were also expressed in *Xenopus* oocytes injected with crab skeletal muscle fibre mRNA (Fournier et al 1990) and two Ca channels were found in patch clamp conditions in the plasma membrane of the tonic flexor muscle of the crayfish (Bishop et al 1991) The activity of both these Ca channels (14 pS and 38 pS) was increased after application of the peptide proctoline

In spite of this interest, data are lacking from simultaneous measurements of membrane and contractile responses of muscle fibres exposed to adrenergic drugs The experiments described were intended to fill-in this gap They demonstrated the importance of both *extracellular* and *intracellular* Ca^{2+} ions for the inotropic effect of adrenaline as well as the modulatory effect of adrenaline on the conductance of the three components of calcium currents described by Henček and Zacharova (1991) in the crayfish muscle fibre membrane

Preliminary results of these experiments have been reported shortly elsewhere (Lipskaja et al 1985, Lipskaja and Zacharova 1987, Zacharova et al 1993)

Materials and Methods

All experiments were performed on single muscle fibres dissected from the middle part of the m extensor carpopoditi of the crayfish *Astacus fluviatilis* After dissection the isolated fibre was fixed in a measuring chamber to determine its slack length (l_0) and the maximum and minimum diameters in two mutually perpendicular axes The diameter was measured at least at 10 points along the entire length of the fibre One tendon of the fibre was fixed, the other attached to a hook made from fine silver wire, connecting the

fibre to a silicon tensiometer (Marko et al. 1986) for recording the isometric tension. The fibre was stretched to 120% of their slack length before the beginning of the experiment.

Recording of electrical and mechanical responses

Intracellular stimulation was performed and electrical membrane responses were recorded by means of Ling–Gerard capillary microelectrodes (Bureš et al. 1967). The recording microelectrodes were filled with 3 mol/l KCl (10–15 MΩ) and the stimulating ones with 2 mol/l K citrate (3–5 MΩ). Duration of stimulating pulses was 30–70 ms. Membrane potentials and contractile responses were displayed on a storage oscilloscope (5103N Tektronix, USA) and simultaneously photographed from the screen. Temperature of the bath was maintained at the required value (as a rule 20 °C) by means of a thermistor controlled cooling system.

Caffeine contractures were recorded in a perfusion chamber, which enabled to exchange the superfusion medium in fraction of a second. Caffeine solutions (6 mmol/l) were prepared by dissolving the drug in normal crayfish saline (van Harreveld solution) of the following composition (in mmol/l): Na^+ 208.4; K^+ 5.4; Ca^{2+} 13.5; Mg^{2+} 5.6; Cl^- 248.8; HEPES 10 to keep pH at 7.3–7.5.

Recording of calcium currents

The vaseline gap voltage clamp method (Hille and Campbell 1976) was used to record Ca currents in muscle fibre segments. The segments were prepared by cutting the tendon ends of the fibre in internal solution of the following composition (in mmol/l): 240 Cs-glutamate; 1.0 $MgCl_2$; 0.01 Ca-glutamate; 10 EGTA; 5 ATP; 0.2 cAMP; 10 HEPES; pH 7.3. The muscle fibre segment was quickly transferred to the experimental chamber and positioned over partitions in the chamber. Vaseline strips were applied over the fibre by means of a syringe through a 350 μm blunted needle. The detailed experimental set-up has been described elsewhere (Zahradník and Zachar 1987).

Calcium ionic currents were recorded after analog compensation for the leak and capacitance components and filtration with a 10 kHz low pass filter. A home made data acquisition software was used to store data for a later analysis. Dissection of individual current components (differing in their time courses of activation and inactivation) from total calcium current, was based on the Hodgkin-Huxley model for conductances (Hodgkin and Huxley 1952). Exponent 6 instead of 3 in the H-H equation for m variable was used for calculation of the calcium conductance (Henček and Zachar 1977). In order to evaluate amplitude a time parameters of a particular component the currents were transformed to conductances (mS/cm^2) assuming an equilibrium potential of + 50 mV. The time course of the calcium conductance, g_{Ca} was fitted by summing two or three equations with six or nine parameters:

$$g_{Ca} = g_f\,(1 - \exp(-t/\tau_{mf}))^6 \exp(-t/\tau_{hf}) + g_i\,(1 - \exp(-t/\tau_{mi}))^6 \exp(-t/\tau_{hi}) + \\ + g_s\,(1 - \exp(-t/\tau_{ms}))^6 \exp(-t/\tau_{hs}),$$

where g_f, g_i, g_s are conductances of the individual calcium current components; and τ_{mx}, τ_{hx} are the corresponding time constants of activation and inactivation, where x stands for f, i and s respectively. The area under the time course of a particular conductance was evaluated by numerical integration.

Calcium currents were recorded in the following solution (in mmol/l), which superfused the tested membrane area: 208.3 Cs-glutamate or TMA-glutamate and 13.5

Ca-glutamate, pH 7.3. Adrenaline chloride (6.10^{-6} – 6.10^{-5} mol/l) was added to this solution just before the experimental procedure.

Measurement of ^{89}Sr fluxes

Fluxes of radioactive ^{89}Sr ions in single muscle fibres were determined by essentially similar procedures as used by Hodgkin and Horowicz (1959) and Curtis (1966). Single isolated muscle fibres were held in forceps stretched to 120% of their slack length. The forceps were attached to an overhead mechanism which permitted the fibre to be moved rapidly from the loading chamber to the measuring chamber. The experimental set-up enabled a precise positioning of the fibre 10 μm from the bottom in the measuring chamber. The middle part of the fibre was located over a hole (7 or 10 mm in diameter) in a brass plate, which covered the top of a scintillation counter enclosed in a heavy lead shield. The position of the fibre was carefully adjusted to a required distance from the bottom of the chamber with a horizontal microscope. The fibre was then transferred to the loading chamber with radioactive ^{89}Sr solution. The walls of the influx cell were covered with platinum plates, which served for stimulation of the fibre along its whole length. Contractions were observed visually under a second (vertical) microscope. The influx period lasted 20–30 min. The radioactive saline was washed out and the influx cell perfused for 1 min with non radioactive saline. The fibre was then transferred to the measuring cell without crossing the solution-air interface. In the measuring cell the fibre was perfused (10 ml/min) with a non radioactive saline. With the shutter in the brass shield window open the radioactivity remaining in the fibre was measured at regular intervals during the whole duration of efflux lasting 60–80 min. The influx of ^{89}Sr was determined by means of Equ. (Hodgkin and Horowitz 1959):

$$m_i = \frac{\Delta y}{2\pi \cdot r \cdot \tau \left(1 - e^{-t'/\tau}\right)}$$

where Δy is the increase in radioactivity of ^{89}Sr, t' is the duration of influx period, τ is the time constant, and r is radius of the fibre.

The efflux curve can be described by two time constants τ_1 and τ_2. τ_1 is a fast time constant lasting $\approx$ 1–10 min; the slow time constant, τ_2 lasts $\approx$ 150–400 min.

The Sr^{2+} ions were used in concentration of 27 mmol/l replacing the Ca^{2+} ions (13.5 mmol/l) in normal crayfish saline.

Loading of the fibre with ^{89}Sr was usually repeated 3–4 times, so as to bracket the testing influx periods with control flux periods. We examined in this way the effect of Ni^{2+} ions on ^{89}Sr fluxes and modulation of this effect by adrenaline (6.10^{-6} – 6.10^{-5} mol/l).

At the end of experiment the fibre was quickly washed in isotonic Tris-HNO_3 buffer in order to wash-out the extracellular ions. The fibre was then desiccated and the radioactivity of ^{89}Sr remaining in the fibre determined. The radioactivity of the standard loading solution was determined in the same way. All measured values were corrected for the background.

Where appropriate, the Student's t-test was used for statistical evaluation.

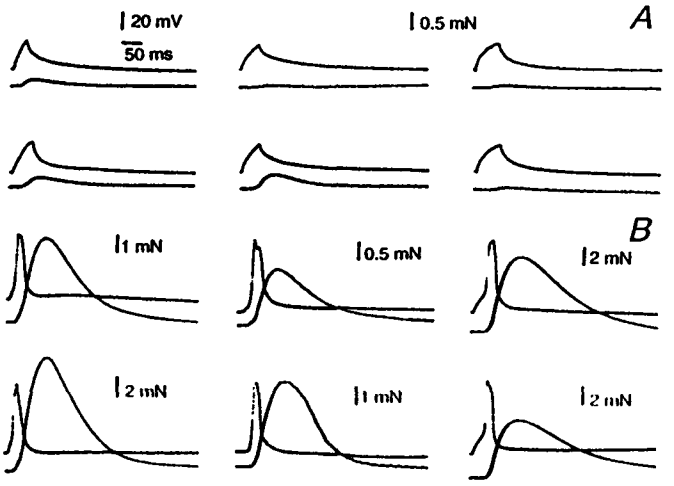

Figure 1. The effect of catecholamines on electrical (*upper beam*) and mechanical responses (*lower beam*) in single muscle fibres of the crayfish. Panel *A*: graded electrical responses and twitches in crayfish saline (van Harreveld solution); first row: controls; second row: the effect of catecholamines (adrenaline – 1 column; noradrenaline – 2 column; isoprenaline – 3 column) added to the crayfish saline. Panel *B*: strontium action potentials and twitches in strontium containing solutions (13.5 mmol/l Ca^{2+} replaced with 27 mmol/l Sr^{2+}); first row: controls; second row: the effect of catecholamines added to the strontium containing solutions (adrenaline – 1 column; noradrenaline – 2 column; isoprenaline – 3 column). Concentration of catecholamines: 6.10^{-6} mol/l. Records taken 5 min after application of the test solution. T = 20 °C. Different single muscle fibres of the crayfish *Astacus fluviatilis*.

Results

The effect of catecholamines on stimulated membrane and contractile responses

In the first series of experiments on single crayfish muscle fibres we compared the effect of catecholamines differing in the type of adrenergic receptor (α, β) they are using for mediating the final effect (Ahlquist 1948). Fig. 1. shows typical effects of adrenaline, noradrenaline and isoprenaline applied at the same concentration of 6.10^{-6} mol/l on simultaneous records of active membrane responses and accompanying twitches. Both adrenaline and noradrenaline potentiated the twitch tension (panel *A*: records on the left and in the middle of the second row; compare with control records in the first row). Isoprenaline (record on the right) did not influence the twitch. A similar effect was observed also after transformation of graded electrogenesis to all-or-none action potentials in strontium solutions (Fatt and Ginsborg 1958); after replacement of Ca^{2+} ions (13.5 mmol/l) with Sr^{2+} ions (27 mmol/l). As found by Zacharová et al. (1962) the contractions in these solu-

tions are potentiated as well. As evident from panel B, the twitches in strontium solutions were potentiated both by adrenaline and noradrenaline (records on the left and in the middle of the second row; compare with control values in the first row in panel B). Isoprenaline was without effect also in this case (record on the right). According to classical categorization this type of agonist action is characteristic for α adrenergic receptors. Changes in contraction characteristics (potentiation of the twitch tension, enhancement of the rate of rise and mainly of the rate of relaxation) are, however, typical for β adrenergic type of responses.

Fig. 2 documents the behavior of 5 parameters; in addition to those described above, also the decrease in threshold depolarization and shortening of latency of

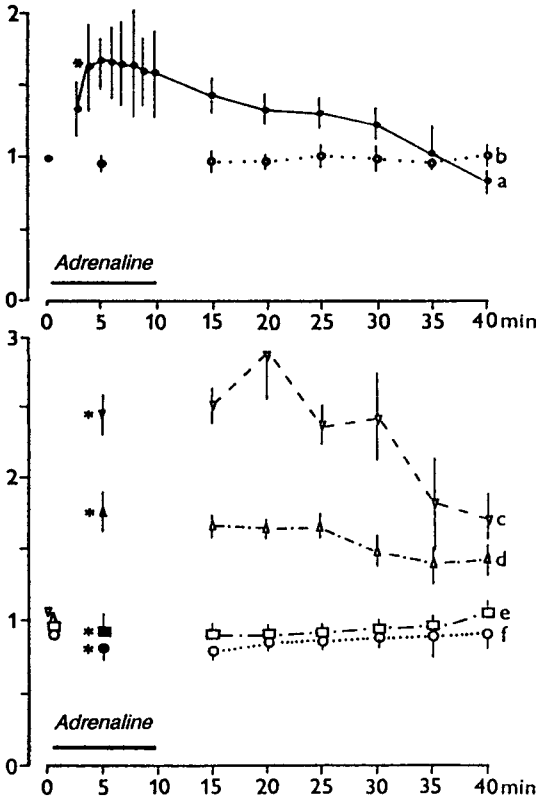

Figure 2. Changes in electrophysiological and contractile parameters after application and withdrawal of adrenaline from the external saline. a – amplitude of twitch tension; b – amplitudes of graded electrical membrane responses; c – rate of rise in twitch tension; d – rate of decay in twitch tension; e – threshold depolarization; f – latency of the twitch. Ordinate in relative units. Abscissa – time (in min) from the moment of application of the test solution. Adrenaline was applied (adrenaline) for 10 min. S.E.M. (n = 10 – 22); * statistical significance (P = 0.05 – 0.001).

the twitch. The maximum effect is reached already in the 5th min after application of adrenaline (6.10^{-6} – 6.10^{-5} mol/l) and the effects persist (mainly the rate parameters of the twitch) for more than 30–40 min after returning to control saline. Changes in twitch parameters in strontium solutions are similar to those described above. The twitch amplitude is increased by 45%, the rate of rise of

Table 1. The effect of adrenaline (6.10^{-6} mol/l) on membrane parameters

	Control	n	Test solution	n
Resting potential	77.7 ± 0.7	83	77.4 ± 1.9	15
AP amplitude	83.3 ± 0.8	143	83.2 ± 1.5	20
AP duration	25.4 ± 0.8	90	24.3 ± 1.2	21
GR amplitude	36.4 ± 1.0	64	40.0 ± 1.8	22

Notes: AP – action potential in strontium solutions; AP duration was determined at 0.5 of the action potential amplitude; GR – graded responses in normal crayfish saline

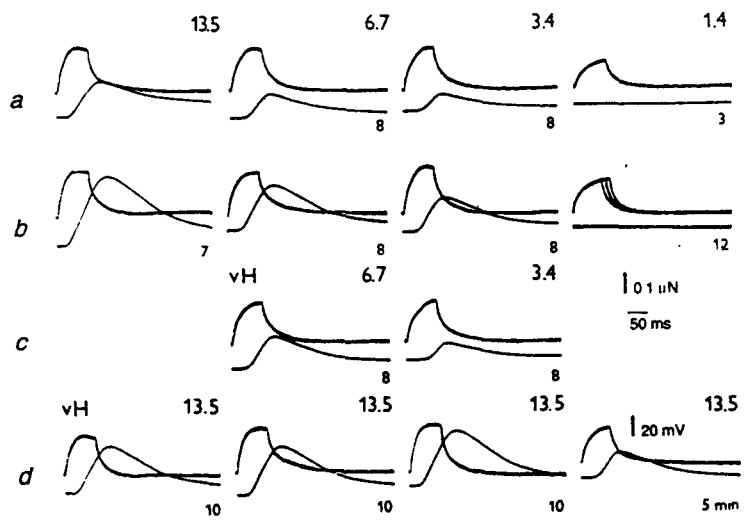

Figure 3. The inotropic effect of adrenaline in relation to the extracellular concentration of Ca^{2+} ions, $[Ca]_0$. $[Ca]_0$ is indicated (in mmol/l) at top right of each record. *a*: control graded electrical membrane responses (upper beams) and accompanying twitches (lower beams) in crayfish saline (vH – van Harreveld solution) with different $[Ca]_0$ (in mmol/l): 13.5 (physiological concentration); 6.7; 3.4 and 1.4. The numbers under the records indicate the time of recording (in min) after application of the test solution. *b*: as in (*a*), but after application of adrenaline (6.10^{-5} mol/l). *c*: recovery of membrane and twitch responses from the effect of adrenaline after switching back to the corresponding control solutions (with $[Ca^{2+}]_0 = 13.5$, 6.7 or 3.4 mmol/l). *d*: recovery from the effect of adrenaline in van Harreveld solution (i.e. in $[Ca^{2+}]_0 = 13.5$ mmol/l).

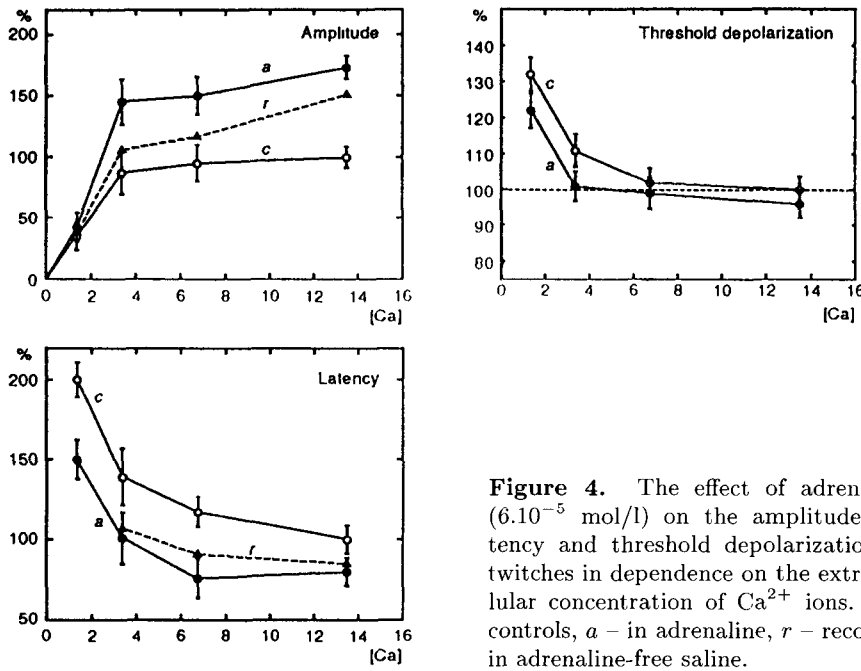

Figure 4. The effect of adrenaline (6.10^{-5} mol/l) on the amplitude, latency and threshold depolarization of twitches in dependence on the extracellular concentration of Ca^{2+} ions. *c* – controls, *a* – in adrenaline, *r* – recovery in adrenaline-free saline.

tension by 53.4%, the rate of relaxation of tension by 184% (n = 15; all changes are statistically significant). The membrane parameters did not change, however, significantly in adrenaline solutions, as follows from Table 1.

Dependence of positive inotropic effect of adrenaline on $[Ca^{2+}]_0$

The excitation-contraction link in muscle fibres of the crayfish is dependent on the extracellular calcium ions (Zacharová and Zachar 1967; Valko et al. 1967) with ensuing intracellular step formed by calcium induced calcium release as recently proved convincingly by Györke and Palade (1992). When the extracellular concentration of calcium ions was decreased, the electrical and contractile responses were changed as shown in Fig. 3. The rate of rise of the electrical response was decreased, the twitch latency was prolonged, the threshold depolarization was increased and the twitch amplitude decreased. Average changes in amplitude, latency and threshold depolarization for initiation of twitches in dependence on $[Ca^{2+}]_0$ are shown in Fig. 4. The amplitude of the twitch is significantly changed already at ten times lower $[Ca^{2+}]_0$ (1.3 mmol/l). After 8 min in adrenaline saline (6 μmol/l) all parameters of the membrane active responses and contraction are facilitated. Though the twitch amplitude in 3.4 mmol/l $[Ca^{2+}]_0$ was facilitated, the after-effect of adrenaline was absent at this $[Ca^{2+}]_0$ in recovery solution (Fig. 3; third row, third column), but present at $[Ca^{2+}]_0$ = 13.5 mmol/l in recovery (van Harreveld) solu-

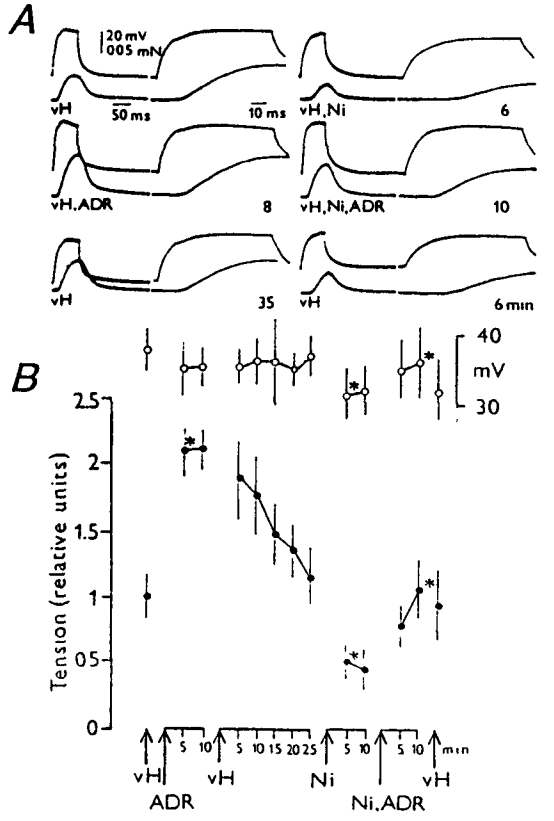

Figure 5. The alleviating effect of adrenaline on graded membrane responses and accompanying twitches depressed by Ni^{2+} ions (1 mmol/l). Note double recordings at two recording speeds (the second one at 5 times higher speed) to show the effect on the rate of rise of the response. Panel *A*. First column: first row – controls in van Harreveld solution (vH); second row – 8 min after superfusion with adrenaline solution (vH, ADR); third row – 35 min after reintroduction of van Harreveld solution (vH). Second column: first row – control in van Harreveld solution containing Ni^{2+} ions (vH, Ni)(6 min); second row – 10 min after superfusion with adrenaline solution containing Ni^{2+} ions (vH, Ni, ADR); third row – 6 min after reintroduction of van Harreveld solution (vH). Panel *B*: summary of experiments as shown by individual records in Panel A. Hollow circles: amplitudes of membrane responses; filled- in-circles: relative twitch tension. The sequence of solution exchanges is shown below the abscissa. Asterisks denote statistical significance ($P = 0.01$).

tion (Fig. 3; fourth row, third column). We observed a similar effect of adrenaline after blocking the influx of Ca^{2+} ions with Ni^{2+} ions (Fig. 5). The rate of rise of twitch tension was decreased in 1 mol/l Ni^{2+} solutions; and the twitch latency

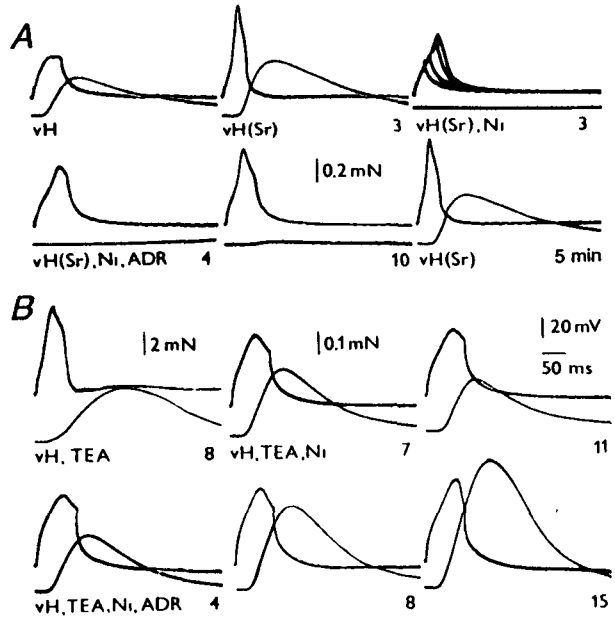

Figure 6. The alleviating effect of adrenaline on strontium action potentials and accompanying twitches (panel *A*) and on TEA (tetraethylammonium) spikes and accompanying twitches depressed by Ni^{2+} ions (1 mmol/l) (panel *B*). The succession of records is from left to right in the first row and continued from left to right in the second row in each panel. The meaning of symbols which denote application of a new external solution is the same as described in Fig. 5. vH(Sr) denotes van Harreveld solution with Ca^{2+} (13.5 mmol/l) replaced with Sr^{2+} ions (27 mmol/l). The time of recording is indicated bottom right (below the tension records).

correspondingly lengthened from 20.4 ± 2.2 to 35.3 ± 3.5 ($n = 9$). Addition of adrenaline (6 μmol/l) to Ni^{2+} solutions annihilated the effect of Ni^{2+}; latency of the twitch recovered from 35.3 ± 3.5 to 26.8 ± 7.6 ($n = 12$).

A similar positive effect of adrenaline was also observed on twitches initiated by strontium or TEA action potentials; TEA (tetraethylammonium ions in 20 mmol/l concentration) (Fig. 6). The overshooting spike was changed to a graded response by Ni^{2+} ions and the accompanying twitch was correspondingly decreased (decrease of AP from 76.8 ± 2.3 to 69.6 ± 2.7 mV ($n = 9$; $P = 0.05$). After addition of adrenaline the spikes and twitches are gradually restored (increase of AP from 69.6 ± 2.7 mV to 74.0 ± 3.5 in the 10 min of adrenaline action); a further increase to 80.3 ± 2.0 was observed after wash-out of adrenaline containing solution. It is evident that adrenaline restores the depressed active membrane responses to original values.

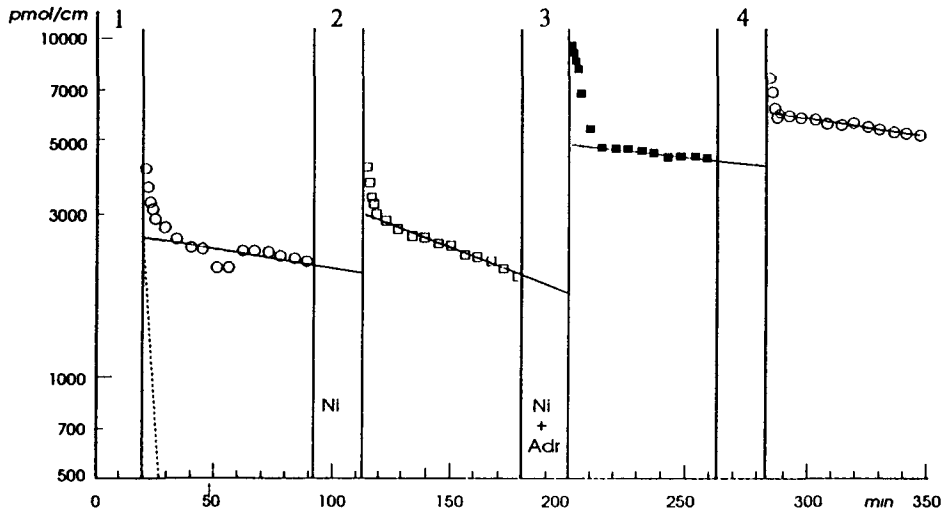

Figure 7. Fluxes of Sr^{2+} ions in 27 mmol/l crayfish saline (van Harreveld solution). The fibre was exposed to ^{89}Sr during 20 min periods (1–4) indicated by the vertical bars. The fibre was stimulated during the influx period along the whole length at 0.1 Hz. The efflux of ^{89}Sr occurs with two time constants, a fast time constant τ_1 (in s; as indicated by the dotted line in the first efflux period) and a slow time constant τ_2 (in min), which was determined from the straight lines through the experimental points. The lines are best fit lines as fitted by least square analysis. Slow time constants are assumed to represent transmembrane ^{89}Sr fluxes. Circles represent the efflux in control saline during the first (1) and the fourth (4) efflux period respectively. Hollow squares: in crayfish saline with added Ni^{2+} ions (the second efflux period; Ni). Filled-in-squares: in crayfish saline containing the Ni^{2+} ions (1 mmol/l) plus adrenaline (6.10^{-5} mol/l); the third efflux period (Ni+Adr).

Fluxes of radioactive ^{89}Sr ions

The supposition that changes in strontium action potential due to Ni^{2+} ions and/or adrenaline might be due to changes in the influx of Sr ions was directly confirmed by following the influx and efflux of labeled ^{89}Sr ions in single muscle fibres. Fig. 7 shows a typical course of the experiment, which lasted for several hours (six hours in this particular case). The vertical lines (1–4) define the 20 min periods during which the fibre was exposed to ^{89}Sr. During the influx periods the fibre was stimulated along its whole length at 0.1 Hz. After the first influx period (1) the muscle fibre was transferred to the measuring chamber to count the number of impulses per min (cpm) from the fibre in flowing crayfish saline. Counting started 2 min after the end of ^{89}Sr influx period and the counts were taken every min at the start of the efflux period (for 10 min) and later every 5th min at regular intervals (see Methods) till the end of efflux period. The efflux curve has a typical course with

Table 2. The effect of Ni^{2+} and adrenaline on stimulated ^{89}Sr fluxes (single muscle fibres of the crayfish *Astacus fluviatilis*)

Solution	Influx (pmol/cm^2/s)	τ_2 of efflux (min)	n
Sr^{2+} saline	24.2 ± 4 7	330.1 ± 59.4	8
Sr^{2+} saline, Ni^{2+}	11.0 ± 2.8*	260.6 ± 34.9	8
Sr^{2+} saline, Ni^{2+}, Adrenaline	20.4 ± 5.8$^{\times}$	381.8 ± 55.9	8
Sr^{2+} saline	16.0 ± 4.2	474.8 ± 124.3	8

Notes: τ_2 – slow time constant of efflux; Sr^{2+} saline contained 27 mmol/l Sr^{2+}; Ni^{2+} (1mmol/l); Adrenaline (6.10^{-5} mol/l). Stimulation frequency: 0.1 Hz. Statistically significant difference against value in the previous row: * $P = 0.02$; $^{\times}$ $P = 0.05$

two time constants: a fast time constant, τ_1 (interrupted line) and a slow one, τ_2 (solid line), which corresponds evidently to efflux from two different muscle fibre compartments. We assume, that τ_1 represents efflux from T-tubular system and invaginations and τ_2 the efflux from the sarcoplasmic reticulum of the fibre.

After 70 min in the efflux cell the fibre was transferred under saline fluid again into the influx cell with added Ni^{2+} ions (1 mmol/l). The other conditions during the second influx period (2; Ni) were the same as in the first influx period, including stimulation and the number of stimuli. The second efflux curve was determined in the same way as during the first efflux period. The gain in tracer, Δy measured as the difference between $y_t\prime$, the quantity of ^{89}Sr at t' ($\approx$ 120th min), and the quantity remaining at time t' from the first exposure is smaller than during the first influx period. It follows, that the Ni^{2+} ions blocked the influx of Sr^{2+} into the fibre.

During the third influx period (3; Ni+Adr) the fibre was stimulated after addition of adrenaline (6.10^{-5} mol/l) to the previous radioactive saline (^{89}Sr saline containing 1 mmol/l Ni^{2+} ions). The gain in tracer was increased in comparison with the solution containing Ni^{2+} alone. Adrenaline evidently counteracts the blockade of Sr^{2+} influx by Ni^{2+} ions.

The fourth influx period (4) represents the final control period in ^{89}Sr crayfish saline.

Average values of the ^{89}Sr influx and values of the slow time constant τ_2 of the efflux curve in Ni^{2+} solutions before and after addition of adrenaline are shown in Table 2; it is evident that adrenaline counteracts the changes in ^{89}Sr fluxes evoked by Ni^{2+} ions.

Average values of the ^{89}Sr influx and values of the time constant of the efflux curve are collected in Table 3. In control experiments with the effect of adrenaline on ^{89}Sr fluxes, i.e. fluxes, which were not inhibited by Ni^{2+} ions, the slow time con-

Table 3. The effect of adrenaline on resting (A) and stimulated (B) ^{89}Sr fluxes (single muscle fibres of the crayfish *Astacus fluviatilis*)

Solution	Efflux		Influx, m_i
	τ_1 (min)	τ_2 (min)	(pmol/cm^2/s)
	A Resting fluxes		
Sr^{2+} saline	4 4 ± 0 7	171 ± 27	15 3 ± 2 4
Sr^{2+} saline, Adrenaline	3 9 ± 0 6	230 ± 43*	12 8 ± 2 0
Sr^{2+} saline	3 6 ± 0 8	208 ± 45	16 3 ± 2 7
	B Stimulated fluxes		
Sr^{2+} saline	3 5 ± 1 3	324 ± 95	18 3 ± 5 3
Sr^{2+} saline, Adrenaline	3 7 ± 1 3	370 ± 89*	11 6 ± 3 4*
Sr^{2+} saline	1 9 ± 0 5	285 ± 130	24 0 ± 8 3

Notes τ_1 – fast time constant of efflux, τ_2 – slow time constant of efflux, Sr^{2+} saline contained 27 mmol/l Sr^{2+}, Adrenaline ($6\ 10^{-5}$ mol/l) Stimulation frequency 0 1 Hz Statistically significant difference against value in the previous row * $P > 0\ 05$, $n = 8$–10

stant of the efflux curve was prolonged significantly both in resting and stimulated fibres, the influx was significantly depressed in stimulated fibres.

It follows, that adrenaline modulates the influx of Sr ions depending on the actual conductance of the Ca channel

The effect of adrenaline on calcium release from the sarcoplasmic reticulum

In the following series of experiments we tried to test the assumption, whether the positive inotropic effect of adrenaline in crayfish muscle fibres is due only to an increased influx of Ca ions from the extracellular environment of the cell, or to an enhanced mobilization of Ca ions from sarcoplasmic reticulum as well, i.e. analogously as in well documented heart muscle cells (for a review see Reiter 1988, Hartzell 1988). We have used procaine, which is known to block the release of calcium from the sarcoplasmic reticulum and caffeine, which on the other hand facilitates calcium release from the SR (Feinstein 1963, Antoniu et al. 1985, Weber and Herz 1968, Endo 1977, Su and Hasselbach 1984, Simon et al. 1989) The effect of procaine in crustacean muscle fibres is doublefold. First of all it transforms graded membrane responses to full-grown action potentials (procaine spikes), in view of the fact that it blocks more effectively the potassium channels than the calcium channels in the membrane (Hagiwara et al 1969) In spite of potentiated membrane responses, the contractile responses are depressed, both evoked by caffeine (Uhrík and Zacharová 1970, 1976) or by electrical stimulation (Suarez-Kurtz and Sorenson 1976, Zacharová et al 1985).

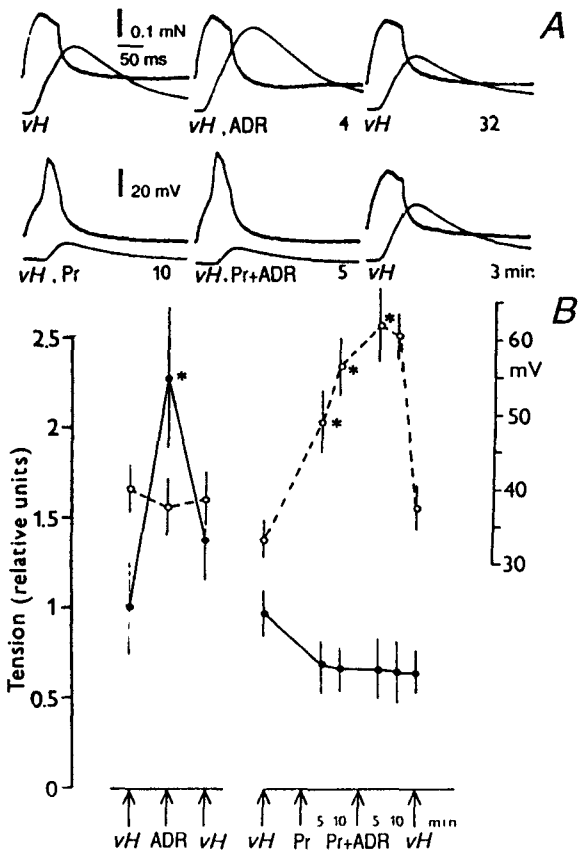

Figure 8. The effect of adrenaline (6.10^{-6} mol/l) on the procaine action potential and the accompanying contraction. Panel *A*: First row shows the effect of adrenaline and recovery after adrenaline withdrawal in a control series. Second row demonstrates the generation of an all-or-none spike; recorded in the 10th min after addition of procaine (2 mmol/l) to crayfish saline. Note the absence of twitch tension changes after addition of adrenaline to the procaine saline (vH, PR, ADR), and the fast recovery of twitch tension in van Harreveld solution. Panel *B*: summary of experiments as shown by individual records in Panel A. Hollow circles: amplitudes of membrane responses; filled-in-circles: relative twitch tension. The sequence of solution exchanges is shown below the abscissa. Asterisks denote statistical significance ($P \geq 0.05$).

Fig. 8 shows the effect of adrenaline on procaine spike and accompanying contraction. The first row in panel A shows the typical positive inotropic effect of adrenaline in the absence of procaine. After application of procaine (2 mmol/l) the graded membrane response was changed to an all-or-none spike (first record in the second row; vH, PR), but the twitch tension was depressed. After application

of adrenaline (second row, the record in the middle; vH, PR, ADR) the amplitude of the action potential was increased, but the twitch tension did not change at all. The recovery in control crayfish saline (vH) was complete. Average changes in action potential and contraction under the influence of procaine are demonstrated in panel B. It follows from the experiments first, that adrenaline in concentrations leading to positive inotropic effect is unable to influence dissociation of excitation-contraction link induced by procaine; and second, that also calcium released from sarcoplasmic reticulum is needed for the positive inotropic effect of adrenaline to occur.

It is now well documented that caffeine acts by inducing calcium release from and inhibiting calcium uptake by the sarcoplasmic reticulum both in cardiac and skeletal muscle. On the contrary stimulation of β adrenergic receptors and G_s proteins enhances the uptake of calcium into sarcoplasmic reticulum. The question, whether adrenaline acts in the same way in crayfish muscle fibres, was therefore addressed by testing its effects on caffeine contractures as well. Caffeine contractures can be evoked repeatedly in single muscle fibres of the crayfish with a relative stable amplitude if a sufficient interval is observed between individual caffeine application, if the caffeine concentration is not maximal and its time of application is short (Zachar 1971).

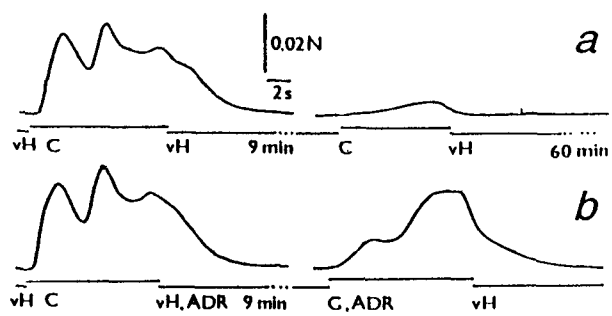

Figure 9. The effect of adrenaline on caffeine (6 mmol/l) contractures. Records of two caffeine contractures (C) evoked in succession after a 9 min interval in the absence (a) and in the presence (b) of adrenaline (6.10^{-6} mol/l) in contracture solution.

Fig. 9 shows the effect of adrenaline (6.10^{-6} mol/l) on the ability of caffeine (6 mmol/l) to induce tension in the refractory phase after a conditioning contracture. The conditioning contracture was cut short after 10 s by reintroduction of caffeine-free saline. In a new trial (after 9 min) to induce a second caffeine contracture, only a small contracture tension could be generated (recordings in the first row). When the experiment was repeated in solutions containing adrenaline (6.10^{-6} mol/l),

the fibre was able to generate substantial contracture tension also to the second testing trial The average testing contractures (as measured by the area under the contracture tension) were significantly higher (55.0 ± 12% of the conditioning contracture) in adrenaline than in control crayfish saline (29 5 ± 9%) (n = 6; P = 0 05)

The effect of adrenaline on calcium currents, I_{Ca}

Adrenaline does increase calcium currents both in heart (Reuter and Scholz 1977) and in skeletal muscle (Arreola et al 1987) The effects of adrenaline in crayfish muscle fibres are demonstrated in Fig 10, which shows 4 types of responses we

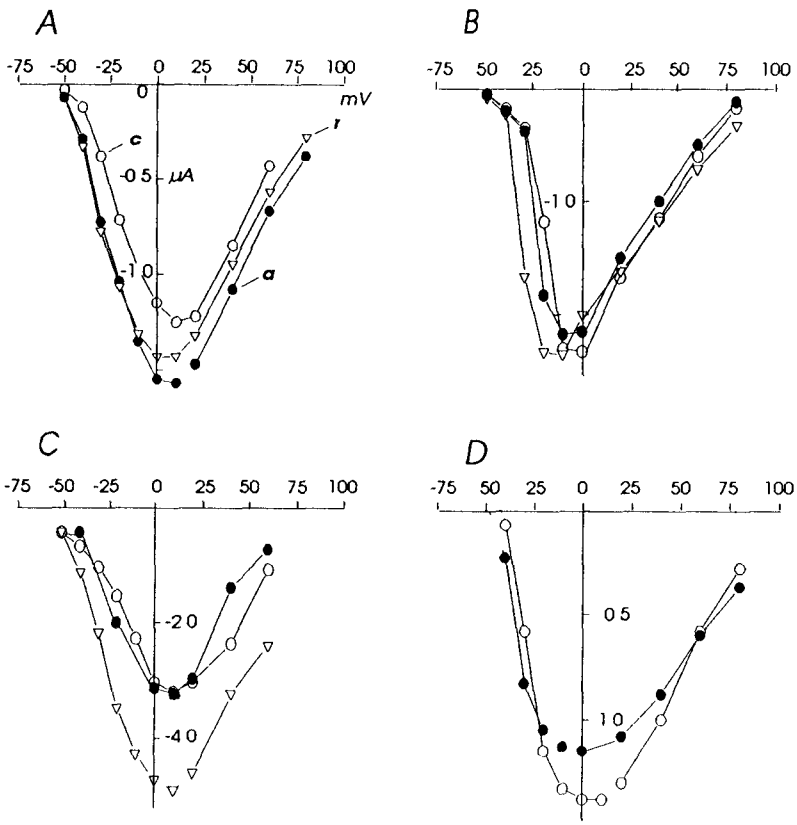

Figure 10. The effect of adrenaline (6 µmol/l) on peak inward currents recorded in voltage clamp conditions (vaseline-gap method) in four (A D) different muscle fibre segments of the crayfish, representing 4 different types of responses (for further explanation see text) Hollow circles control solutions, filled-in-circles after addition of adrenaline (5–10 min) to the external saline, triangles recovery in normal crayfish saline Holding potential, HP = −80 mV

have observed in 12 internally perfused muscle fibre segments. The A-type is characterized by an increase of Ca currents in the whole range of applied voltages and by persistence of these changes after wash-out of the adrenaline. The B-type shows enhancement of Ca currents at lower depolarizations, which becomes more apparent after reintroduction of adrenaline-free saline. The C-type is similar to the B-type, except the enhancement of Ca currents in the whole range of voltages after the withdrawal of adrenaline from the external saline. In the D-type the Ca currents are increased at lower depolarizations (−50 to −20 mV) and then decreased around the maximum of the I–V curve. This type of adrenaline effect was also characteristic for the current-voltage relation in strontium solutions (4 segments – not shown). It seems, that the most constant effect of adrenaline on Ca currents in crayfish muscle fibres is represented by increase of I_{Ca} at lower depolarizations and shift of the I–V plot to the left.

If the recording was performed in TMA glutamate solutions (TMA^+ replacing monovalent cations) containing 13.5 mmol/l Ca glutamate, it was possible to record at low depolarizations Ca curents, which inactivated quickly and resembled to calcium currents in T-type calcium channels. The inactivating components appeared by increasing smoothly the membrane depolarization – usually by 10 mV steps. When stepping further a third component on the inactivation phase of the fast channel became visible, which resembled the third type of calcium channel (the N-type) (Henček and Zacharová 1991).

Fig. 11 shows the effect of adrenaline (6 μmol/l) in a fibre with clearly expressed fast (g_f) and slow component (g_s) respectively of the total calcium conductance (g_{Ca}). The left column shows control conductances at three membrane depolarizations −30, −10 and 0 mV, which can be compared with calcium conductances after application of adrenaline (6 μmol/l) (right column). The main change concerns the slowly inactivating calcium conductance (denoted by s). The value of the fast inactivating component (denoted by f; T-type) changes very little, but the rate of inactivation is prolonged.

Fig. 12 shows the effect of adrenaline (6 μmol/l) in a fibre exhibiting all three calcium conductance components, fast (g_f), intermediate (g_i) and slow (g_s) component respectively. It is evident that adrenaline already in the 2nd min after application prolongs the inactivation of the fast component (g_f), increases the conductance of the intermediate component (g_i), and as a main effect, the conductance of the slow component (g_s).

The effects of adrenaline on calcium conductances in muscle fibres of the crayfish are summarized in Table 4, and the recovery after adrenaline withdrawal in Table 5. The maximum effect of adrenaline is already reached 2–5 min after application at membrane depolarization of −30 mV, and remains so at higher depolarizations as well. The fast calcium conductance is increased by ≈ 38%, the intermediate conductance by about 100% and the slow calcium conductance by

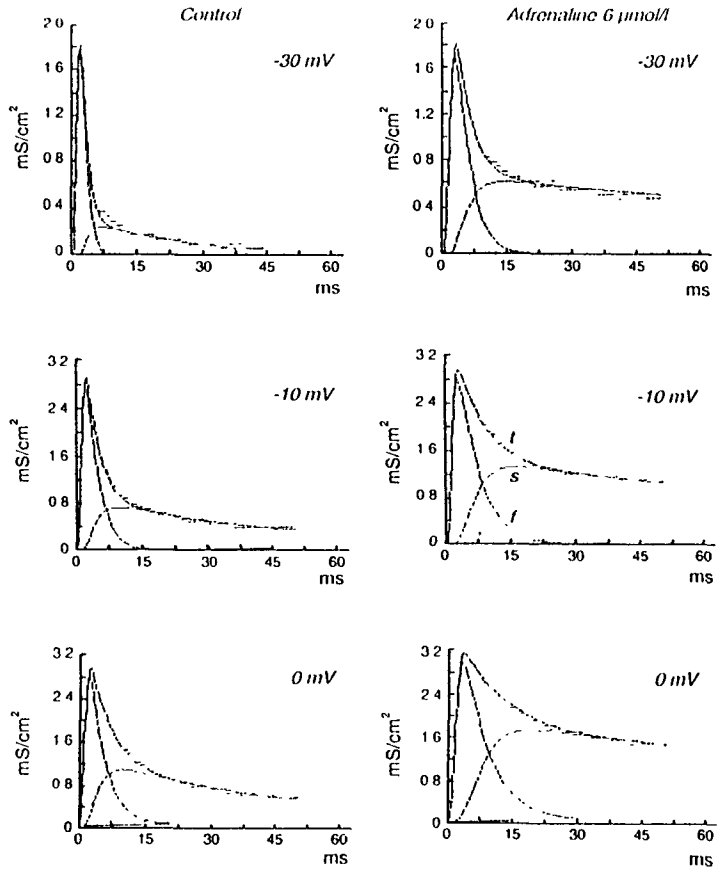

Figure 11. The effect of adrenaline on calcium conductances g_{Ca} (in mS/cm^2) in crayfish muscle fibre segments. The component calcium conductances, g_f (fast), and g_s (slow) were dissected from the total calcium conductance g_{Ca} using H–H equations as described in text. The time courses of the total g_{Ca}, g_f and g_s are denoted by t, f and s respectively. The ionic currents were evoked by a series of depolarizing pulses to membrane depolarizations −30, −10 and 0 mV starting from the holding potential of −80 mV. Left column: controls in van Harreveld solution; right column: after addition of adrenaline (6 μmol/l). Holding potential; $HP = -80$ mV.

138%. The changes are similar, if the conductances are evaluated from the areas under the conductance curves or conductance amplitudes respectively (compare two last rows in Table 4). The effect of adrenaline persists after the withdrawal of adrenaline and might be even increased; it mainly concerns the intermediate and the slow calcium channel respectively. Significant changes in kinetic parame-

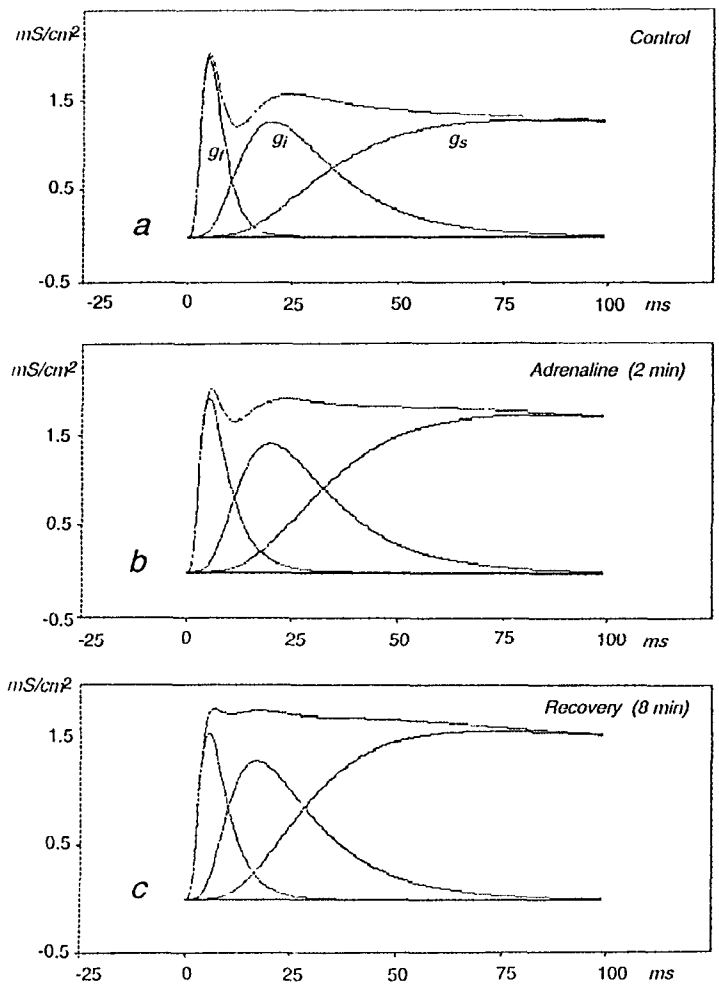

Figure 12. The effect of adrenaline on the time course of the individual calcium conductance components (in mS/cm^2), i.e. g_f (fast), g_i (intermediate) and g_s (slow) respectively. The total calcium conductance is represented by the uppermost curves in all three panels. The upper panel: calcium conductances in control saline; the middle panel: 2 min after addition of adrenaline to the saline (6 μmol/l); the lower panel: 8 min after reintroduction of control van Harreveld solution. Holding potential; membrane depolarization: -30 mV; $HP = -80$ mV.

ters were encountered only in the time constant of fast channel inactivation, which decreased from $\tau_h = 1.0$ to 1.29 ± 0.09 ($n = 6$); $P = 0.05$.

Table 4. The effect of adrenaline on calcium conductance components in single muscle fibres of the crayfish *Astacus fluviatilis* (in % of the control values)

MD(mV)	t(min)	g_f	g_i	g_s	n
−30	2– 5	120.9 ± 8.8	194.5 ± 23.6*	213.7 ± 42.5*	10
−30..0	2– 5	129.5 ± 7.4	173.2 ± 15.4*	218.3 ± 29.1**	17
−30..0	2–11	131.8 ± 6.3	201.5 ± 20.9**	232.2 ± 27.6§	22
−30..0	2–11	116.6 ± 5.2	209.4 ± 28.9**	232.3 ± 23.8§	22†

Notes: fast (g_f), intermediate (g_i), and slow (g_s) calcium conductance components; MD – membrane depolarization; t – time interval after application of adrenaline; † evaluated from maximal amplitudes of conductances; all other values from the area under the conductance curves. Statistically significant difference against g_f; * $P \geq 0.05$; ** $P \geq 0.01$; § $P \geq 0.001$

Table 5. The effect of adrenaline withdrawal on calcium conductance components in single muscle fibres of the crayfish *Astacus fluviatilis* (in % of the control values)

Solution	g_f	g_i	g_s	n
Adrenaline	149.7 ± 11.8	272.7 ± 56.4	263.5 ± 61.9	6
Recovery	158.2 ± 38.4	308.5 ± 54.7	333.5 ± 77.6	6

Notes: fast (g_f), intermediate (g_i), and slow (g_s) calcium conductance components; Adrenaline (6 μmol/l) – values recorded 8-11 min after application of adrenaline; Recovery in normal crayfish saline – values recorded 7–9 min after adrenaline withdrawal.

Discussion

Progress in knowledge on the effects of β-adrenergic substances on heart muscle always preceded that in skeletal muscle cells. This was mainly due to the prevalent clinical concern in heart muscle, on one hand, and to the structural properties of these muscle groups, on the other. The patch- clamp technique applied to cardiomycytes enabled to study intensively the classical β-adrenergic cAMP pathway and the underlying second messenger cascade, finishing with phosphorylation of Ca channels. Every step has been documented in myocardial cells by several methods (Hille 1989). It was therefore of interest to compare the results obtained in heart muscle with the effects of β-adrenergic stimulation in crustacean skeletal muscle cells, whose calcium channels show similar electrophysiological properties (Henček and Zachar 1977, Zahradník and Zachar 1983, Györke and Palade 1992) as those in heart muscle cells. It mainly concerns higher rates of activation and inactivation of

Ca channels in heart myocytes (for a review see Peltzer et al. 1992) in comparison with calcium channel rate parameters in skeletal muscle fibres of vertebrates (for review see Beaty et al. 1987). It seems that also the α_1-subunit of the L-type Ca channel, which was isolated from the skeletal muscle of the crayfish (Križanová et al. 1990), resembles more to that of cardiac DHP receptor (Cooper et al. 1987), than to the α_1-subunit of the L-type Ca channel in vertebrate skeletal muscle (Flockerzi et al. 1986), at least as far the binding properties and molecular weights are concerned. The crayfish muscle contains, however, at least subunit homologous to the respective proteins from mammalian skeletal muscles; and the protein with molecular mass of approximately 190 kD was phosphorylated specifically with cAMP dependent protein kinase (Hudecová and Križanová 1991).

The excitation-contraction coupling mechanism in heart and crustacean skeletal muscle and namely the calcium induced calcium release (Fabiato and Fabiato 1978, for review see Callewaert 1992; Ashley et al. 1991 and Györke and Palade 1992, 1993) represents another distinct common trait in these muscle types. In skeletal muscle fibres of vertebrates, on the other side, a mechanical interaction between the DHP receptors and the calcium release channel of the sarcoplasmic reticulum is supposed to form basis of the E-C coupling mechanism (Schneider and Chandler 1973; for review see Ríos et al. 1991). The Ca release channel of the SR (the ryanodine receptor) was isolated, purified and reconstituted in all three muscle types; i.e. in vertebrate skeletal muscle (Inui et al. 1987; Hymel et al. 1988a, b), in heart muscle (Anderson et al. 1989), as well as in crustacean muscle (Formelová et al. 1990; Seok et al. 1992) and localized in feet structures at the apposition of T system tubules and membranes of the SR. In both groups of muscle fibres with Ca induced Ca release mechanism (cardiac muscle fibres of chicken and arthropod skeletal muscle respectively) the junctional proteins of the tubular membrane are not grouped in tetrades (as in skeletal muscle fibres of vertebrates), and do not seem to bear a precise relationship to the feet (Loesser et al. 1992; Franzini-Armstrong and Takekura 1993).

In spite of several common features, the calcium channel types display a kind of tissue specificity (for review see Lory et al. 1991; Tsien et al. 1991; Miller 1992) and recent studies have shown molecular and functional diversity of G_s stimulated adenylcyclases (for a review see Iyengar 1993; Eschenhagen 1993).

As a first step in comparative studies we tried therefore to get basic data on the effect β-adrenergic agonists, and mainly adrenaline, in skeletal muscle fibres from m. extensor carpopoditi of the crayfish, which we used for a long time as an experimental object, and are fairly well characterized functionally, morphologically and biochemically (for a review see Zachar 1971, 1981; Zachar and Zacharová 1989; Palade and Györke 1993).

It follows from the presented results, that the natural adrenergic agonists, adrenaline and noradrenaline induce in muscle fibres of the crayfish a positive ino-

tropic effect, in contrast to the synthetic β-adrenergic agonist isoprenaline, which is ineffective at the same concentration (6 μmol/l). This contrasts with the effect of β-adrenergic synthetic agents isoprenaline and isoproterenol in cardiac myocytes, where the synthetic compounds are more effective in low concentrations and as such are therefore frequently used in the inquiry into the mechanism of action of β-adrenergic substances (for a review see Reiter 1988, Pelzer et al. 1990).

The effects of adrenaline on contractile responses in crayfish muscle fibres (the amplitude of twitch and tetanic tension; the rate of rise and decay of twitch tension) are similar to the effects of adrenaline in the heart (Morad and Rolett 1972; for a review see Hartzell 1988) and the skeletal muscle fibre of the frog (Oata and Nagai 1977; Gonzales-Serratos et al. 1981; for a review see Williams and Barnes 1989b). The characteristics of the β-adrenergic response support the assumption on the existence of β-adrenergic receptors in skeletal muscle fibres of the crayfish, which are also functional in conditions of cultures media (Zacharová et al. 1990b). This supposition is also supported by the effect of the β-adrenergic blockator propranolol on the adrenaline effect (Zacharová et al. 1988). Propranolol did block the positive inotropic effect of adrenaline, similarly as in frog skeletal muscle (Oata and Nagai 1977); but propranolol did also block the calcium channels, similarly as in neurons of *Aplysia* (Akaike et al. 1982). Thus the direct blocking effect of propranolol on the Ca channel activity induced by adrenaline cannot be excluded. The binding of adrenaline to the receptor should initiate the whole cascade of events, i.e. an increase in adenylcyclase, cAMP and cAMP-dependent proteinkinase A, and consequently also the phosphorylation of the Ca channel. The experiments with forskolin, (a direct activator of the catalytic subunit of adenylcyclase), have shown, that forskolin exerts a qualitatively similar effect as does the adrenaline; the effect of adrenaline was, however, at the same concentration more pronounced (Zacharová et al. 1993). Mediation of the adrenaline effect via the second messenger system–cAMP, is also supported by our preliminary experiments (not yet published) with external application of permeable cAMP analogues (dibutyryl cAMP and 8-bromo-cAMP), which showed a positive inotropic effect of these substances, as well as increase in the rate parameters of the twitch tension.

The effect of adrenaline was concentration-dependent with threshold value of about 1 μmol/l. The sigmoidal relationship resembled that found by Huddart and Battram (1984) in crab muscle activated by nerve stimulation. The same concentration dependence of the inotropic effect of adrenaline was also observed in strontium solutions, i.e. after a change of graded membrane responses to all-or none spikes. The time lag to reach the maximum inotropic effect (5 min) is comparable to that observed in single skeletal muscle fibres of the frog (Oata and Nagai 1977). The time of onset of the inotropic effect in intact fibres correlated well with the onset of changes in calcium currents, recorded from muscle fibre segments. The same applies for the persistence of positive inotropic effect on contraction and of

enhanced calcium channel conductance after the wash-out of adrenaline (Fig. 12 and Tab. 5). The correlation between the inotropic effect of adrenaline and the enhanced influx of Ca^{2+} ions via calcium channels may be taken as evidence for the dependence of the inotropic effect on the extracellular calcium.

The negative inotropic effect induced by calcium antagonists in myocard is completely eliminated by activation of β-adrenergic receptors (Fleckenstein 1977). We have observed the same effect of adrenaline also in muscle fibres of the crayfish. Adrenaline annihilated the blocking effect of Ni^{2+} ions on both the membrane and contractile responses and increased the influx of $^{89}Sr^{2+}$ ions, which was suppressed by Ni^{2+} ions. The underlying mechanism of this effect might be ascribed in both cases to protein phosphorylation-mediated reaction which results in an increase in Ca^{2+} or $^{89}Sr^{2+}$ uptake via the Ca channel and thus a stimulation of contractility. Similarly as in intact and skinned muscle fibres of the frog (Endo et al. 1970; Cognard and Raymond 1985) the Sr^{2+} ions are able to induce calcium release from the SR in crustacean muscle fibres as well (Stephenson and Williams 1980; Goblet and Mounier 1987) and could be accumulated in the sarcoplasmic reticulum (Tomková and Kontšeková 1980). The capacity of sarcoplasmic reticulum vesicles to accumulate strontium is about 20% lower than calcium. The accumulation of strontium in lateral cisternae of the SR in muscle fibres of the frog have been demonstrated by X-ray microanalysis (Uhrík and Zacharová 1988).

Four types of current-voltage relationships, we have observed, point to a variability, not unusual in the experimental conditions of our experiments (internally perfused single muscle fibre segments). The most frequent type is characterized by the increase of calcium currents in the range of lower depolarizations. Negative shift in the peak of the $I_{Ca} - V$ relation was observed in ventricular myocytes by several authors (Fischmeister and Hartzell 1986; Bean 1989; Peltzer et al. 1990). The mechanism of β-agonists effect on a single channel level resides in the increase of opening probability of the Ca channel at a constant unitary channel current amplitude as first described by Reuter et al. (1983), as well as in the increase of the number of active channels (Reuter and Scholz 1977), as verified by fluctuation analysis in myocytes (Bean et al. 1984). The latter authors also showed that the enhancement of peak current decreases gradually with greater membrane depolarizations. The decrease of Ca currents at higher depolarizations, from -20 mV, (type 4) was most frequent ($\approx$ 50%) at physiological concentration of Ca^{2+} ions (13.5 mmol/l) and constantly present (100%) after replacement of Ca^{2+} with Sr^{2+} ions (27 mmol/l). Negative shift (5–10 mV) of Sr currents after application of adrenaline was a constant phenomenon at threshold depolarizations.

The decrease of I_{Ca} or I_{Sr} at higher depolarizations can be explained by different assumptions. It could be due to the "run-down" of calcium channels, which is often observed in internally perfused cells (for a review see Hagiwara and Byerly 1981; Kostyuk 1984). The recovery to control values after wash-out of adrenaline

in several muscle fibre segments is, however, difficult to reconcile with the above assumption. It is also possible that the influx of Ca^{2+} ions into the fibre can inactivate the calcium channel by a rise in the intracellular level of Ca^{2+} ions (Ca induced inactivation of the L-type channel; Kokubun and Irisawa 1984; Lee et al. 1985; Kass and Sanguinetti 1984; Tseng 1988); and switching the channel operation to a mode in which the channels would access the open state rarely. The decrease of Ca ionic currents was also observed, when higher concentrations of isoproterenol (10^{-6}) were used (Reuter et al. 1983). When measuring the influx of labelled $^{89}Sr^{2+}$ using the doubled concentration of divalent cations (13.5 mmol/l of Ca^{2+} replaced with 27 mmol/l of Sr^{2+}) we frequently observed a decrease of influx of $^{89}Sr^{2+}$ upon application of adrenaline (6.10^{-5} mol/l), which attained control values after exchanging the adrenaline solution for normal crayfish saline (see Tab. 3). When the activity of Ca channels was decreased in Ni^{2+} solutions, adrenaline on the contrary, increased the reduced influx of $^{89}Sr^{2+}$ ions. It seems that a definite optimal level of depolarization and/or concentration of adrenaline exists, which leads to an increase of calcium currents.

The total ionic current from muscle fibre segments of the crayfish shows (after replacement of all permeable cations (excepting Ca^{2+}) with TMA and anions with glutamate in external solution) two distinct current components in Ca^{2+} solutions (Zahradník and Zachar 1982, 1987) and in some segments even three current components (Henček and Zacharová 1991), which can be differentiated by the activation threshold, by the rate of activation and first of all by the time course of inactivation. Individual calcium current components and conductances can be dissected from the total calcium current by means of Hodgkin-Huxley equations (see Fig. 11 and Fig. 12; for details see Methods). The first component, which inactivates fairly quickly is supposed to represent calcium current through a fast calcium channel (T-type channel). The slowest component which activates and inactivates very slowly corresponds to an L-type channel current. The third (intermediate) component similar to the N-type calcium channel current shows slower inactivation in comparison with the fast component. Adrenaline enhanced strikingly (> 100%) the calcium conductance of the slow and the intermediate calcium channel. The calcium conductance of the fast channel was increased only by about 30% and its rate of inactivation was decreased. The effect of β-adrenergic agents on the T-type of calcium channel in heart muscle is controversial; it seems, however, that recently new evidence was added, which supports the T-channel as a target of β-ergic as well as α-ergic agents (for review see Peltzer et al. 1992; Alvarez and Vassort 1992).

Potentiation of the slow and the fast calcium channel by adrenaline and cAMP in skeletal muscle fibres of the frog was described by Arreola et al. (1987) and Stefani et al. (1987). The latter authors have observed similar effect also after application of α-adrenergic agonist phenylephrine. The fast calcium channel observed in adult frog skeletal muscle (Cota and Stefani 1986) shows, however, a different

kinetics in comparison with the T-channel in the heart (Bean 1989) and crayfish muscle (Zahradník and Zachar 1987, Henček and Zacharová 1991). Like T current in cardiac and crayfish muscle it activates with a low threshold and very rapidly; unlike T current it does not inactivate over several seconds. This current is not sensitive to dihydropyridines.

The fast channel in crayfish muscle is influenced both by adrenaline and by organic blockators (Zacharová et al. 1990a, 1993; Henček et al. 1993). The effect of these agents on the L-type channel is, however, more pronounced. Hence the differences are qualitative only. Our results obtained in voltage-clamp conditions from the whole cell membrane are supported by the results of Bishop et al. (1991), who demonstrated in abdominal muscle fibres of the crayfish enhancement of two types of calcium channels after application of a pentapeptide proctolin in patch-clamp conditions. The conductance of one channel was 14 pS and of the other, a slow one was 36 pS. The value of 14 pS in other structures characterizes, however, rather the N-type channel (Bean 1989). The conductance of the calcium channel isolated from the sarcotubular fraction of the crayfish muscle and reconstituted in lipid bilayers was 16 pS (Hurňák et al. 1990). This channel was activated by the dihydropyridine activator BAY 8464; the effect of adrenaline was not tried. Nifedipine and diltiazem inhibit in crayfish muscle all calcium current components at the concentrations (1–10 μmol/l) (Zacharová et al. 1990a; Henček et al. 1993). It is not possible therefore to distinguish between the N-type or the fast channel. After withdrawal of calcium ions from the extracellular medium the fast calcium conductance is substantionally decreased after few seconds. The intermediary calcium conductance is much less affected, and the slow calcium conductance is facilitated. After 1–2 min all Ca conductances disappeared. After reintroduction of Ca containing solution the fast calcium component was the first to recover. We therefore suppose (Henček and Zacharová 1991), that the fast calcium channel is localized in the surface membrane and might perform a similar function as the Na channel in skeletal muscle or Na channel in heart muscle, which are responsible for generation and propagation of action potential in excitable cells. In crab muscle Brule et al. (1987) found a quick disappearance of the fast component of contraction after the withdrawal of Ca ions and suppose that this component of contraction is due to activation of fast calcium channel. A similar assumption was put forward by Morad and Cleemann (1987) in heart muscle; the role of the T-channel might be in triggering the calcium release mechanism from the SR. If we take into account the new results showing that the cardiac Na channels might be enhanced by β-adrenergic stimulation (Tytgat et al. 1990; Matsuda et al. 1992) the positive modulation of the fast Ca channel by adrenaline in our experiments does not seem surprising.

In addition to the positive inotropic effect of adrenaline accompanied by an increased influx of Ca^{2+} ions, we also observed the second mechanical effects of adrenaline, i.e. accelerated rates of contraction and relaxation.

The cell cAMP increased by β-ergic stimulation does influence the second internal membranous system – the sarcoplasmic reticulum – as well; by increasing the Ca^{2+} fluxes in this system (Fabiato and Fabiato 1975; Allen and Blinks 1978). cAMP accelerated Ca uptake by ATPase-phospholamban system can explain the acceleration of relaxation because the increase rate of Ca uptake by SR would increase the rate at which Ca is removed from troponin (for review see: Tada and Katz 1982; Tada and Kadoma 1989).

We examined two aspects of β-adrenergic stimulation of SR in muscle fibres of the crayfish. First, the enhancement of the Ca-induced Ca release and the dependence of the inotropic effect on this mechanism. Second, the enhancement of the reuptake of Ca^{2+} into SR; tested by means of pharmacological agents which either block (procaine) the Ca release channel or release Ca^{2+} ions from the SR (caffeine). Procaine (2–8 mmol/l) blocked contraction as well as the positive inotropic effect of adrenaline, in spite of the fact that the action potential evoked by procaine was increased by adrenaline (both the amplitude and the rate of rise of AP). Procaine itself, as we have shown earlier (functionally and morphologically), blocked caffeine contractures (Uhrík and Zacharová 1970, 1976), in spite of the fact that the calcium conductance changed very little (Zacharová et al. 1985) as found in voltage-clamp conditions on intact and in internally perfused muscle fibres of the crayfish. Procaine depressed both types of K channels (Ca activated and delayed rectifier).

An increase in caffeine contractures after application of octopamine was demonstrated in the whole muscle of the crayfish by Fisher and Florey (1987), a fact which points to an increased release of calcium from the SR by this neurotransmitter. Our results showed in addition an increased reuptake of Ca^{2+} into SR as proved by testing the duration of refractoriness of caffeine contractures in the absence and in the presence of adrenaline (Fig. 9).

The mechanical effect of adrenaline can take place only after refilling calcium in the SR. If the influx of Ca^{2+} was lowered, either by decreasing the extracellular $[Ca^{2+}]$ or by blocking the Ca channels by Ni^{2+} ions, the positive effect is present, but dependent on the actual (i.e. depressed) control contraction. The persistence of the inotropic effect after the wash-out of adrenaline (the post-effect) is, however, absent. If the extracellular $[Ca^{2+}]$ is increased to physiological values, the "post-inotropic effect" reappears (Fig. 3*d*). The increased force of contraction after application of adrenaline may be explained as a result of both increased influx and increased mobilization of internal calcium; by a similar mechanism as was shown to work in heart muscle (for a review see Hartzell 1988).

Binding of adrenaline to the receptor and ensuing intramembranous and intracellular events mediated by G_s proteins are independent on the extracellular $[Ca^{2+}]$. The mechanical effect of adrenaline, as well as the contraction itself is dependent on the extracellular $[Ca^{2+}]$ and on the amount of calcium in the SR. In

the resting state the increased accumulation of calcium in the SR due to increased influx of calcium can be also maintained by a decreased efflux of calcium from the SR. It follows from our experiments with radioactive ^{89}Sr which showed that the time constant of efflux of ^{89}Sr from the fibre was prolonged after application of adrenaline and after its removal.

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