The Effect of Pb²⁺ Ions on Calcium Currents and Contractility in Single Muscle Fibres of the Crayfish

D. ZACHAROVÁ, M. HENČEK, J. PAVELKOVÁ and E. LIPSKÁ

*Institute of Molecular Physiology and Genetics, Slovak Academy of Sciences,
Vlárska 5, 833 34 Bratislava, Slovakia*

Abstract. Muscle fibres of crustaceans represent an useful model for studying the mode of action of substances influencing calcium channels as the membrane generates the active responses on a pure calcium principle and the excitation – contraction link is dependent on external calcium. We followed the effect of Pb²⁺ ions (1–300 μmol/l) on contractile responses in single muscle fibres of the crayfish evoked by massive or intracellular electrical stimulation, by potassium depolarization and caffeine application, as well as on action potential in single intact muscle fibres and on calcium currents in voltage clamp conditions (vaseline gap) in internally perfused muscle fibre segments. All types of contractile responses, single twitches, tetanus, potassium and caffeine contractures were blocked. The strontium action potential was blocked very effectively by Pb²⁺ ions. The total calcium currents which can be split by means of Hodgkin-Huxley equations into two components (fast and slow respectively) differing in the rate of activation and inactivation were suppressed after addition of $Pb(NO_3)_2$ (50-300 μmol/l). The effect of Pb²⁺ was concentration and time dependent. At lower concentrations (100 μmol/l) the blocking effect was more pronounced on the fast inactivating Ca current component. The Pb²⁺ ions prolonged the time constant of inactivation τ_h of the slow channel, while leaving that of the fast channel intact.

Key words: Pb ions — Calcium currents — Fast and slow calcium channels — Contraction — Potassium contractures — Caffeine contractures — Muscle fibre — Crayfish muscle — Calcium dependent electrogenesis — Hodgkin-Huxley equations

Introduction

It was shown recently that the Pb²⁺ ions block calcium channels in neuronal membranes of both vertebrates as well as invertebrates (Audesirk 1987, Audesirk and Audesirk 1989, 1991; Büsselberg et al. 1990, 1991; Evans et al. 1991). The blocking concentration of Pb²⁺ ions was lower for L and N channels activated at higher membrane depolarizations in comparison with T channels activated at lower mem-

brane potential displacements. Evans et al. (1991), comparing the effect of lead ions on Ca, Na and K channels in cultures of ganglion cells from dorsal roots of the rat, have shown that the Pb^{2+} ions block selectively calcium channels, and namely at concentrations which are toxic in human pathology.

The aim of the present experiments was to find out, if and how the Pb^{2+} ions influence the contractility and excitability in crustacean muscle fibres, which generate active membrane responses on calcium principle (Fatt and Ginsborg 1958) and comply a calcium dependent E-C coupling mechanism (Zacharová and Zachar 1967). The muscle membrane system of the crayfish *A. fluviatilis*, which was used in these experiments, posses two types of calcium channels, i.e. a fast inactivating (T-type) and a very slowly inactivating (L-type) calcium channel (Zahradník and Zachar 1982, 1987; Jdaïâa and Guilbault 1986; Bishop et al. 1991). Recently even three calcium current components were observed in crayfish muscle fibres (Henček and Zacharová 1991).

The Pb^{2+} ions were shown to block both types of calcium channels; the block of fast channel was, however, more pronounced at lower concentrations of lead ions. The Pb^{2+} ions prolonged the time constant of inactivation (τ_{hs}) of the slow Ca channel leaving the time constant of inactivation of the fast Ca channel (τ_{hf}) unchanged.

Materials and Methods

The experiments were performed on both intact single muscle fibres and internally perfused muscle fibre segments dissected from 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 and the maximum and minimum diameters. A small silver hook fixed to one fibre tendon served to connect the fibre to a silicon tensiometer (Marko et al. 1986) in the experimental chamber. The chamber allowed a rapid exchange of solutions bathing the fibre (Zachar et al. 1964). The fibres were stretched to 120% of their slack length.

Two platinum electrodes covered the lateral walls of the perfusion channel in the experimental chamber which permitted stimulation of the fibre along its whole length. The muscle fibres were stimulated by 2 ms pulses of supramaximal voltage to achieve maximal amplitudes of the twitch and the tetanus (50–100 Hz).

Potassium contractures were induced by sudden application of high potassium solutions (8 or 32 fold) keeping the $[K]_0 \times [Cl]_0$ product constant. Potassium was substituted in exchange for sodium and chloride was replaced with propionate in these solutions. 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.

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).

The vaseline gap voltage clamp method (Hille and Campbell 1976) was used to record Ca currents in muscle fibre segments. The detailed experimental set-up has been described elsewhere (Zahradník and Zachar 1987). The muscle fibres were cut and kept in internal solution of the following composition (in mmol/l): Cs-glutamate 240; MgCl$_2$ 1.0; Ca-glutamate 0.01; EGTA 10; ATP 5; cAMP 0.2; HEPES 10; pH 7.3. The extracellular solution superfusing the tested membrane area contained (in mmol/): TMA-glutamate 208.3 and Ca-glutamate 13.5. Pb(NO$_3$)$_2$ or PbCl$_2$ (1–300 μmol/l) were added from stock solutions to the extracellular solution. pH of Pb solutions in higher [Pb^{2+}] (200–300 μmol/l) was adjusted to 7.1.

Ionic currents were recorded after analog compensation for the leak and capacitance components and filtration with a 10 kHz low pass filter. Data acquisition software including computer driven stimulation was written in the C language by I. Stavrovský in our Institute. Dissection of the individual current components (differing in their time courses of activation and inactivation) from total calcium current, based on the Hodgkin-Huxley model for conductances (Hodgkin and Huxley 1952) in mS/cm^2 assuming the equilibrium potential of 50 mV. 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). The time course of the calcium conductance, g_{Ca} was fitted by summing two equations with six parameters:

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

where g_f, 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 and s respectively. The area under the time course of a particular conductance was evaluated by numerical integration.

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

Results

Contractile responses

Fig. 1 shows changes in the twitch (left panel) and the tetanus tension (right panel) of single muscle fibres of the crayfish after sudden exposures to a crayfish saline containing 1, 5 and 25 μmol/l of Pb(NO$_3$)$_2$. There is a small, but reproducible facilitation of the twitch isometric tension to a concentration 1 μmol/l of Pb^{2+} ions as measured 5 min after a sudden change of normal crayfish saline (van Harreveld solution) for the test solution. This concentration of Pb^{2+} ions ([Pb^{2+}]) represents, however, a threshold for the depression of the tetanus tension. The tetanus tension shows facilitation during recovery from the effects of this [Pb^{2+}]. First signs of twitch depression are evident at [Pb^{2+}] = 5 μmol/l. The twitch tension is also facilitated during recovery from the exposure to this concentration of lead ions. At [Pb^{2+}] = 25 μmol/l both twitch and tetanus tension are clearly depressed during exposure to the test solution; the recovery in the 6th min is not yet complete. The mean values ($n = 3 - 10$) as well as the time course of the *on* and the *off* effects are summarized in Fig. 2. At [Pb^{2+}] = 25 μmol/l (hollow circles) the amplitude of

twitch tension (a) is depressed to 51 ± 1 % of the control value in the 1st min after sudden exposure to the test solution and to 45 ± 6 % in the 5th min respectively. The blocking effect increased with the increase of $[Pb^{2+}]$ (100 and 200 μmol/l). The effect of Pb^{2+} ions on the amplitude and the area under the twitch (a, b) is in parallel with changes in the rate of rise (c) and decay (d) of the isometric tension, which are also time and concentration dependent; this applies also to the recovery of the contractile parameters.

The Pb^{2+} ions block both potassium and caffeine contractures as well, as summarized in Fig. 3 for $[Pb^{2+}] = 200$ μmol/l. Potassium contractures were evoked by increasing the external concentration of K^+ ions ($[K^+]_0$) to 43 and 173 mmol/l respectively at constant $[K]_0 \times [Cl]_0$ product. The interval between application of contracture solutions was equal or longer than 30 minutes. The Pb^{2+} ions were applied 3 min before application of the test contracture solution. Fig. 3 A–C shows that the Pb^{2+} ions block all three measured parameters of the contracture tension in parallel (i.e. the amplitude, the area and the rate of rise of contracture tension). The blocking effect of Pb^{2+} ions on potassium contracture parameters is reversible.
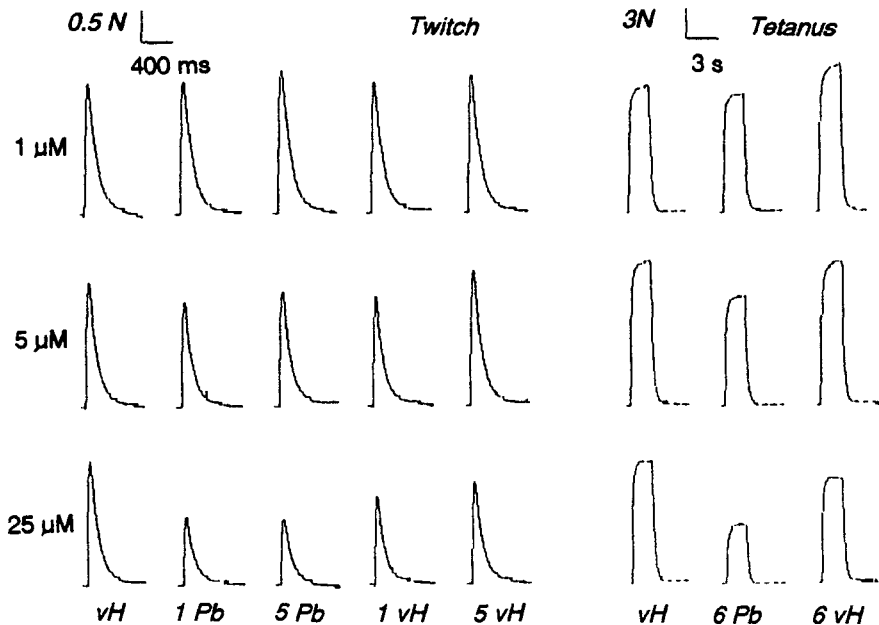

Figure 1. The effect of lead ions (1, 5, 25 μmol/l) on twitch and tetanus tension in single muscle fibres of the crayfish. Supramaximal massive external stimulation with 2 ms pulses. Tetanus frequency: 100 Hz. Contractile responses were recorded at the indicated time (in min) after the application of Pb^{2+} ions or after their withdrawal from the van Harreveld solution (vH).

   Caffeine contractures, which are known to be due to direct release of calcium
ions by caffeine from the sarcoplasmic reticulum, were blocked by Pb^{2+} ions (200
μmol/l) as well (Fig. 3 D–F), although to a lower degree (68 $\pm$ 10 %, $n = 7$,
$p = 0.05$) than the potassium contractures (26 $\pm$ 11 %, $n = 4$). Recovery from
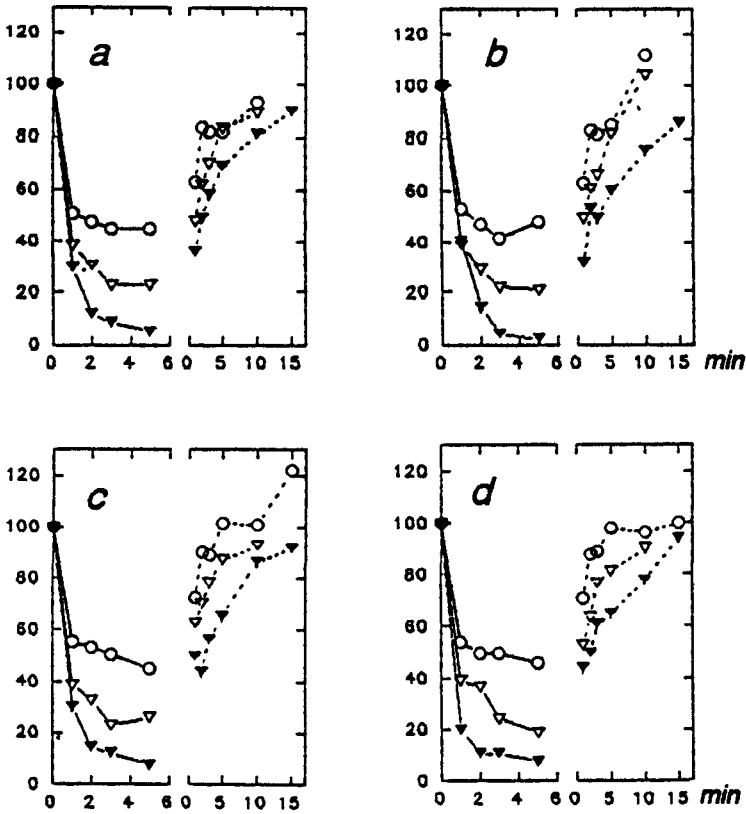caffeine contractures is slower than that from potassium contractures; the interval

Figure 2. The time course of the blocking effect of Pb^{2+} ions. Basic parameters of
the twitch response in relative units (the control parameters were taken as 100 %): *a*)
the amplitude; *b*) the area, *c*) the rate of rise and *d*) the rate of decay of the twitch
tension. Note the time course of reversibility after sudden withdrawal of Pb^{2+} ions from
the crayfish saline. Hollow circles – 25 μmol/l; open triangles – 100 μmol/l; filled triangles
– 200 μmol;l. Individual points represent the means from 3–10 different single muscle fibres
of the crayfish.

between applications of contracture solutions was therefore increased to 60–70 min.

Action potential and contraction

In order to localize the blocking effect of Pb^{2+} ions in the excitation–contraction link, we recorded (in current clamp conditions) electrical membrane responses simultaneously with contraction in two ionic environments, i.e. in normal crayfish saline ([Ca] = 13.5 mmol/l)(3 fibres) and after substitution of Ca^{2+} with Sr^{2+} ions (3 fibres). In strontium solutions the local and graded membrane responses are changed to full grown action potentials (Fatt and Ginsborg 1958) accompanied by augmented twitch tension (Zacharová et al. 1962). The effect of Pb^{2+} ions on the strontium action potential and the accompanying contraction is shown in Fig. 4. The action potential (pulse duration = 40 ms) was completely blocked already 3
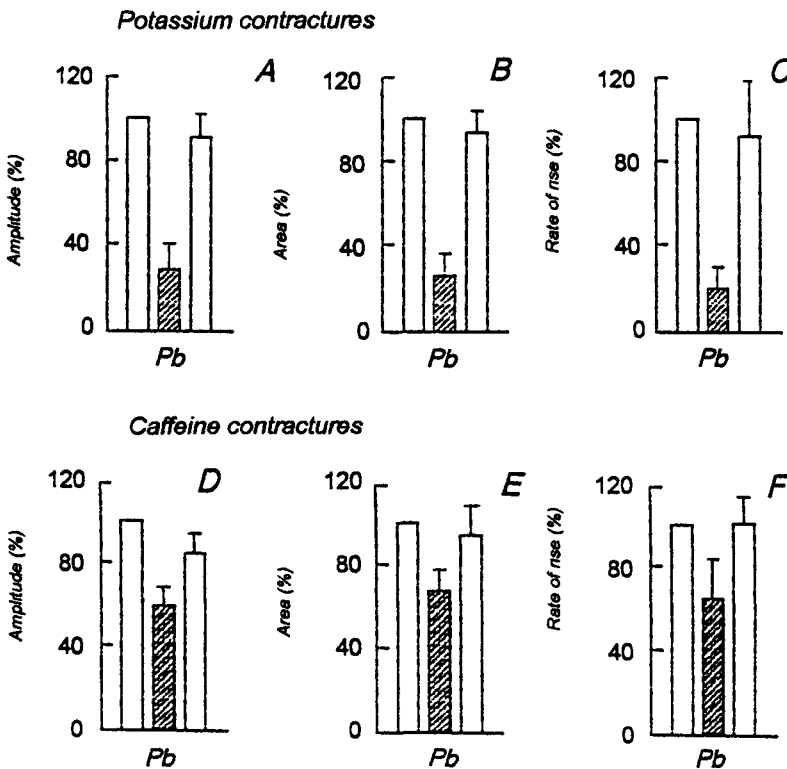

Figure 3. The effect of lead ions on the amplitude (A, D), the area under the contracture (B, E) and the rate of rise (C, E) of potassium and caffeine contractures respectively. The experimental columns represent mean values obtained 3 min after sudden application of the lead containing solutions. The first column in each triad represents control values; the third one the recovery.

min after the application of the testing solution (100 μmol/l). After prolongation of the pulse duration to 80 ms it was still possible to evoke an abortive action potential, which was generated with a delay from the local electrotonic potential (Fig. 4c). The abortive spike decayed with time and was abolished in the 8th min after the application of the test solution (Fig. 4e). The spike was substantially restored 6 min after the withdrawal of Pb^{2+} ions (Fig. 4f), in contrast to the twitch,
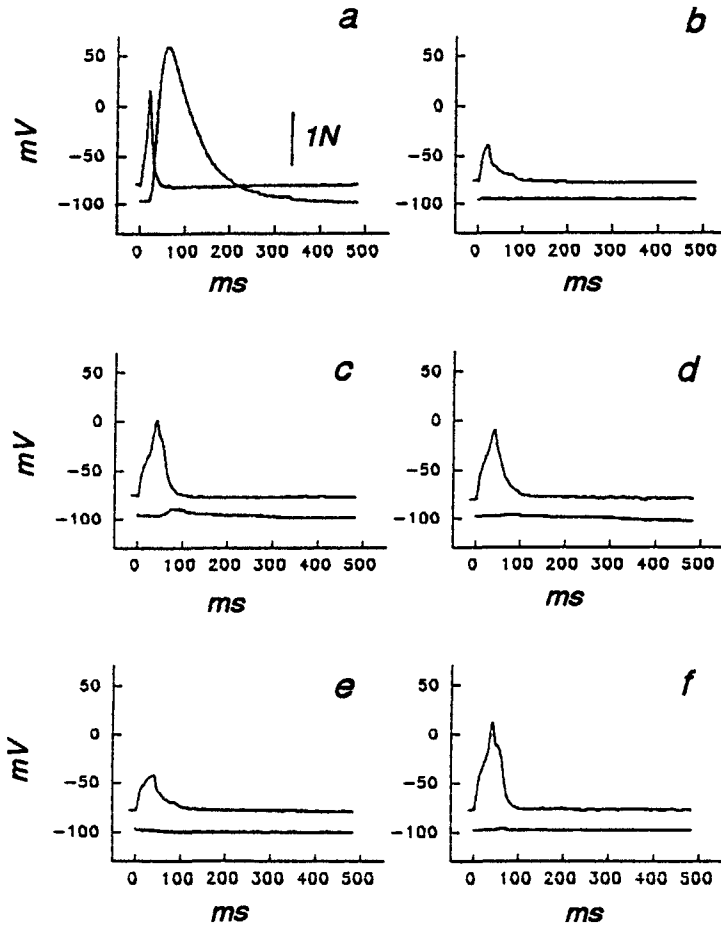

Figure 4. The effect of lead ions on the excitation–contraction coupling. The upper beams represent intracellular records of strontium action potentials (Ca^{2+} ions were replaced with Sr^{2+} ions; 13.5 mmol/l). The lower beams are records of the accompanying twitches. The stimulus duration was 40 ms (a, b) and 80 ms (c – f) respectively. a) control recordings; b) full block 3 min after the introduction of Pb(NO$_3$)$_2$ (100 μmol/l); c), d), e) 4 min, 6 min and 8 min after introduction of the test solution; f) recovery 6 min after the withdrawal of lead ions.
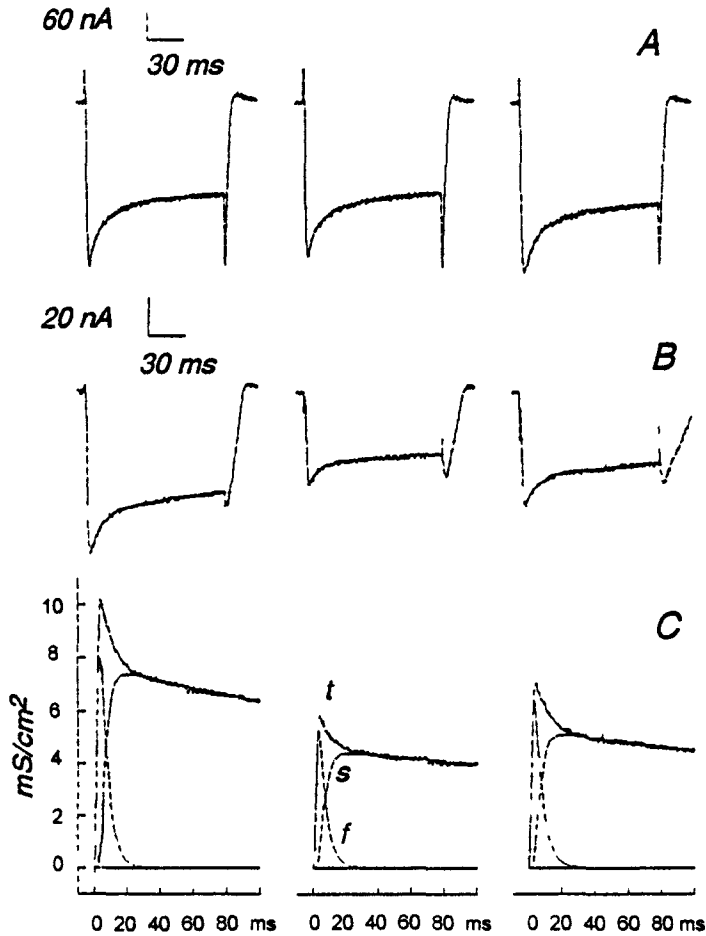
which was still suppressed

Figure 5. The effect of lead ions on calcium currents (A, B) and their dissected fast and slowly inactivating components (C) First row left   control current in 13 5 mmol/l calcium glutamate and 208 TMA mmol/l, middle   5 min after the introduction of Pb^{2+} (50 μmol/l), right – 6 min after the withdrawal of lead ions Second row the effect of 200 μmol/l, the same time parameters as in A, a different single muscle fibre   Third row total (t), fast (f) and slowly (s) inactivating calcium conductances (mS/cm^2) were evaluated (see Methods) from the records in the second row

Calcium currents and conductances

Two types of calcium currents were registered from segments of crayfish muscle fibres in conditions of the vaseline–gap voltage clamp method, i.e. a fast inactivating calcium current which is activated at lower depolarizations (MP = −45 mV) and a slowly inactivating current which is generated at about 10 mV higher membrane depolarizations (Zahradník and Zachar 1982, 1987; Henček and Zacharová 1991).
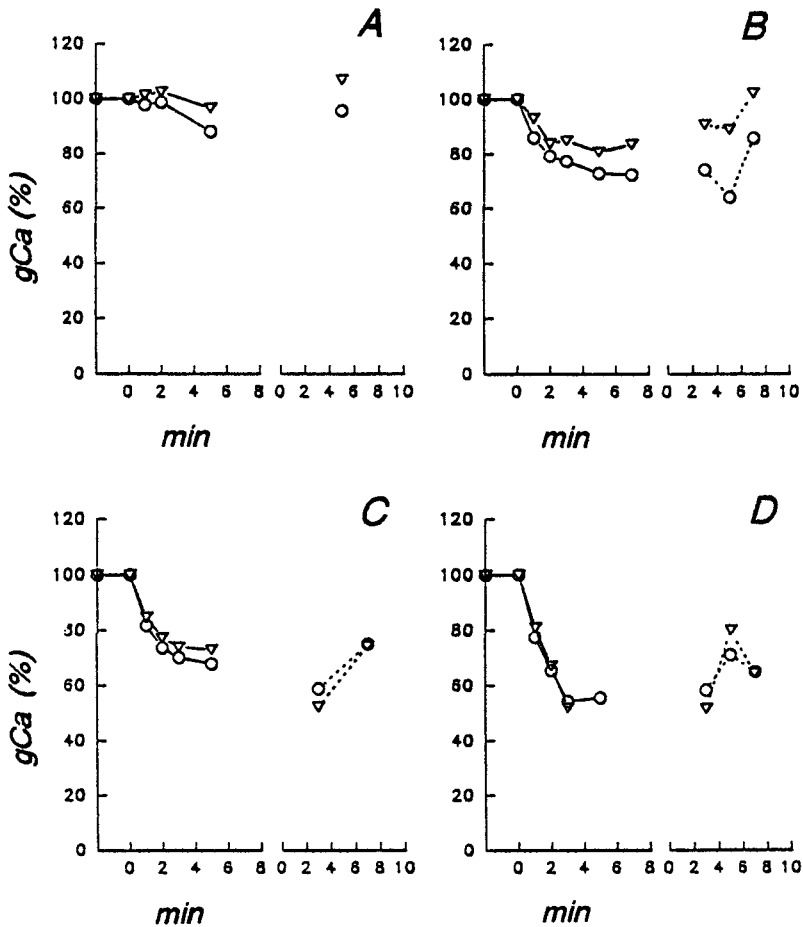

Figure 6. The effect of Pb^{2+} ions on the fast (circles) and the slowly inactivating calcium channel conductances (triangles). Abscissa: time in min after application and withdrawal of the test solutions respectively (note the split abscissa). Ordinate: relative calcium channel conductances. The concentration of Pb^{2+} ions was: *A)* 50; *B)* 100; *C)* 200; *D)* 300 μmol/l respectively. The effect of Pb^{2+} ions (100 μmol/l) on Ca conductances was significantly different in the 3rd minute after application of Pb^{2+} ions ($P > 0.05; n = 8$).

Fig. 5 (A, B) shows records of total calcium currents from internally perfused muscle fibres, when the contribution of all other channels except the calcium channels were eliminated (see Methods). The second column shows the effect of Pb^{2+} ions and the third one the recovery in normal crayfish saline, which can be compared with controls in the first column. The extracellular application of Pb^{2+} ions was limited to 5 min in order to avoid overlap of the lead block with the spontaneous run-down of channels, which may accompany the internal dialysis of the cell (Kostyuk 1982). The $[Pb^{2+}] = 50$ μmol/l (first row) was considered as a threshold concentration; there was a slight facilitation after the withdrawal of Pb^{2+} ions from the external solution. It is to be noted that the threshold blocking concentration is one order of magnitude higher than the threshold blocking concentration of contraction (5 μmol/l). At $[Pb^{2+}] = 200$ μmol/l (Fig. 5B; second row) the depression of both calcium current components was evident and the recovery from the lead poisoning was slower. The dissection of the calcium conductance components from the records shown in Fig. 5B by means of the Hodgkin-Huxley equations (see Methods) is demonstrated in Fig. 5C.

The time course of the blocking effect of Pb^{2+} ions on the fast and the slow calcium conductance components is demonstrated in Fig. 6. Both fast and slow calcium conductances decrease exponentially during the first 3 minutes to a sustained level; all points are significantly different from the control values ($P > 0.05 - 0.001$; $n = 5 - 10$). The rate of decay is concentration dependent. The recovery is complete at low $[Pb^{2+}]$ (50–100 μmol/l) and slowed or incomplete at higher $[Pb^{2+}]$ (200–300 μmol/l).

A significant difference between the blocking effect of Pb^{2+} ions on the fast and the slow calcium conductance was encountered after 3 min in $[Pb^{2+}] = 100$ μmol/l; the fast component was blocked to 77 ± 5 % and the slow one to 85 ± 5 % only ($n = 8$; $P = 0.01$). The most obvious differences between the calcium conductances concern, however, the time constant of inactivation τ_{hs} of the slow conductance component, which slows down in a concentration dependent manner as compared to τ_{hf}. The time constants of activation τ_{mf} and τ_{ms} respectively do not change significantly. The following values of the time constants were found at $[Pb^{2+}] = 300$ μmol/l (3 min after application): $\tau_{mf} = 1.08 \pm 0.05$; $\tau_{ms} = 1.01 \pm 0.01$; $\tau_{hf} = 1.01 \pm 0.02$; $\tau_{hs} = 1.38 \pm 0.1$; $n = 10$; $P > 0.01$.

Discussion

Depression of the nerve-mediated contraction by Pb^{2+} ions and the absence of Pb block to direct stimulation in muscle fibres of vertebrates was taken as evidence that the target structure of the Pb^{2+} block has to be localized to praesynaptic sites in neuro-muscular junction (Kostial and Vouk 1957, Manalis and Cooper 1973; Silbergeld et al. 1974). It was suggested that the lead ions represent competitive

inhibitor of the Ca dependent acetylcholine release (Atchison and Narahashi 1984, Cooper and Manalis 1984, Jackson and Bornstein 1984, Manalis et al. 1984, Picket and Bornstein 1984). In contrast with these results all contractile responses mediated by direct stimulation (electrically or chemically) of single muscle fibres of the crayfish, as follows from our results, were blocked by Pb^{2+} ions. The threshold blocking concentration of electrically stimulated single muscle fibres was even lower than that needed to block the neuromuscular junction in vertebrates especially if the seven times higher concentration of Ca ions in the crayfish saline is considered. These differences can be explained if the type of electrogenic muscle membrane in both cases is taken into the account, i.e. the sodium type in muscle membranes of vertebrates and the calcium type in invertebrates. If the calcium channel is assumed as the target structure of the blocking effect of Pb^{2+} ions, the differences could be explained by a block of Ca channels in presynaptic sites of the neuromuscular junction in vertebrates and a block of Ca channels in surface and tubular muscle membranes of invertebrates.

The results of direct recording the active membrane responses in current and voltage clamp conditions qualitatively support this assumption. The strontium action potentials as well as the calcium ionic currents were blocked by Pb^{2+} ions. The threshold blocking concentration of contraction (5 μmol/l) by Pb^{2+} ions is, however, one order of magnitude lower than that required for blocking the calcium ionic currents (50 μmol/l). This difference could be explained by different conditions, when recording from intact and internally perfused muscle fibres respectively. In the former case the Ca channels are not isolated from the other channels, as takes place in the latter case. The internal solution contained, however, in addition to isolating components (Cs ions and Ca-EGTA buffer) also further components (cAMP, ATP, Mg ions) which prevented distortion of the calcium conductance (Kostyuk 1982).

The contraction of muscle fibres of invertebrates, as well as the excitation-contraction coupling, is dependent on the extracellular calcium ions concentration (Zacharová and Zachar 1967, Ashley 1967, Atwater et al. 1974, Hidalgo et al. 1979). There is also experimental evidence, that the calcium induced calcium release from the SR, participates in the E-C coupling mechanism in muscle fibres of invertebrates, e.g. in barnacle (Lea and Ashley 1989) or in crayfish muscle (Györke and Palade 1992); similarly as in the heart muscle (Fabiato 1983; for a review see Callewaert 1992). It means that contraction of the crayfish muscle fibre is dependent on the influx of Ca ions through the Ca channels in the surface/T-tubule membranes ensuing in the liberation of larger amount of Ca from the sarcoplasmic reticulum (SR). It might be therefore assumed that the Pb^{2+} ions penetrate the muscle membrane and block the calcium release channel in the SR. There are indications that lead can enter the cell through calcium channels in bovine medullary cells (Simons and Pocock 1987) or other types of channels (metal ion

activated channels) as documented in neuroblastoma cells (Oortgiesen et al. 1990), as well as in snail neurons (Osipenko et al. 1992). Oortgiesen et al. (1990) found a slow non-inactivating inward current after superfusion the neuroblastoma cells with Pb^{2+} (in the range of 1–200 μmol/l); this current, however, disappeared in Na-free solutions. Simons and Pocock (1987) have shown that the uptake of Pb^{2+} ions was increased during membrane depolarization induced by potassium ions.

Assuming the entrance of Pb^{2+} ions into the muscle fibres of the crayfish, we could explain the unexpected blocking effect of caffeine contractures by Pb^{2+} ions. Caffeine is known to release the $Ca2+$ ions from the sarcoplasmic reticulum both in vertebrates and invertebrates (for a review see Zachar 1971). The target structure of contractility block by Pb^{2+} ions could be thus the calcium release channel of the SR. We are trying now to test this assumption on skinned fibres and by means of microprobe analysis.

Opposite effects are, however, observed when using organic calcium channel blockators nifedipine and diltiazem; in the same conditions of recording of ionic currents and contraction. The threshold blocking concentration of calcium channels (1–5 μmol/l) is two orders of magnitude lower than that for contraction (Zacharová et al. 1990b; Henček et al. 1993). This discrepancy leaves as yet open the question if the dihydropyridine receptor, which acts as a voltage sensor in the T-SR junction of the vertebrate skeletal muscle (Rios and Brum 1987, Tanabe et al. 1988) is identical with Ca channels in the tubular system (Lamb 1991, 1992) and if this mechanism operates in crayfish muscle at all. The dihydropyrine receptor is localized on the α-subunit of the L-type Ca channel in crayfish muscle as well as shown recently (Križanová et al. 1990) and the ryanodine receptor is also present in the crayfish (Formelová et al. 1990) and in the lobster muscle (Seok et al. 1992). The "sensor-type" of E-C coupling in muscle fibres of the crayfish is supported by the mathematical model of calcium distribution in a muscle fibres during contraction and relaxation (Poledna and Šimurdová 1992). In favor of this assumption is also the time correlated disintegration of T-R junction and disappearance of twitches to electrical stimulation in muscle fibres kept in culture media (Uhrík et al. 1986, Zacharová et al. 1990a). From the quantitative point of view, however, it might be objected, that the contact area of the T-SR junction is to small (about 15% of the sarcolemmal and T-system membranes; Uhrík et al. 1980) in comparison with that in frog muscle fibres (about 67%; Mobley and Eisenberg 1975). If the "sensor-type" mechanism operates in the crayfish muscle as well, it probably does so only in synergy with the Ca induced Ca release mechanism. In that case the Pb^{2+} ions might be expected to influence the voltage sensor as well.

It follows from our experiments that the Pb^{2+} ions belong to the category of the most efficient ionic blockators of calcium channels in membranes of the calcium type of electrogenesis. The Pb^{2+} ions suppress in nerve cells of both vertebrates and invertebrates the fast and slowly inactivating (Audesirk 1987, Audesirk and

Audesirk 1989, 1991, Büsselberg et al. 1990, 1991) as well as N-type channel (in ganglion cells; Evans et al. 1991). In our experiments we found a higher sensitivity of the fast channel to Pb^{2+} ions in comparison with the slow channel; i.e. a block at low concentrations (50–100 mmol/l). On some muscle fibre segments (non documented) with three calcium current components (Henček and Zacharová 1991), we found that the intermediate Ca current component (analogous to the N-type channel) was more sensitive to the Pb^{2+} block than the very slowly inactivating Ca current (L-type channel current).

A specific effect of Pb^{2+} ions concerns the time constant of inactivation τ_h, which was prolonged selectively in the slow calcium channel conductance. The results are in a good agreement with the results obtained on neuroblastoma cells (N1E-115), showing a prolonged inactivation of the L-type channel (Audesirk and Audesirk 1991). The agreement also concerns the absence of changes in activation and inactivation of T and N type channels and their homologies in muscle fibres of the crayfish. As far as the specific effect of divalent cations on different types of calcium channels is concerned, it is known that the Ni^{2+} ions are potent blockers of the fast inactivating Ca channel (T-type) as compared with the Cd^{2+} ions, which block preferentially the slow Ca channel (L-type)(for a review see Peltzer et al. 1990). We have shown recently, that the same holds true for the muscle membrane of the crayfish (Henček and Zacharová 1991). The effect of Pb^{2+} ions on the muscle membrane of the crayfish is, however different, when compared with the neuronal membranes; resembling the effect of Cd^{2+} ions in the latter and that of Ni^{2+} in the former membrane respectively.

The finding that the threshold blocking concentration of calcium channels by Pb^{2+} ions in invertebrate muscle is comparable to the lead poisoning effects in the neuromuscular transmission of vertebrates and namely the finding that also the calcium channels of the E-C link including possibly also the Ca release channel in the sarcoplasmic reticulum might represent targets of the lead effects, necessitates the inquiry into the effects of the Pb^{2+} ions on calcium channels present in various muscle systems in vertebrates.

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