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Membrane Ion Conductances of Mammalian Skeletal Muscle in the Post-Decompression State after High-Pressure Treatment

O. Friedrich[1], **K.R. Kress**[1], **H. Ludwig**[2], **R.H.A. Fink**[1]
[1]Medical Biophysics, Institute of Physiology and Pathophysiology, Ruprecht-Karls-University, INF 326, 69120 Heidelberg, Germany
[2]Institute of Pharmaceutical Technology and Biopharmacy, Section Physical Chemistry, Ruprecht-Karls-University, INF 346, 69120 Heidelberg, Germany

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Abstract. Exposure of excitable tissues to hyperbaric environments has been shown to alter membrane ion conductances, but only little is known about the state of the membranes of intact cells in the post-decompression phase following a prolonged high-pressure treatment. Furthermore, almost nothing is known about high-pressure effects on skeletal muscle membranes. Therefore, we investigated changes to the input resistances, membrane potentials and voltage-gated membrane currents for sodium (I_{Na}), potassium (I_K) and calcium (I_{Ca}) ions under voltage-clamp conditions in enzymatically isolated intact mammalian single fibers following a 3-hr high-pressure treatment up to 25 MPa at +4°C. After a 3-hr 20 MPa treatment, the input resistance was increased but declined again for treatments with higher pressures. The resting membrane potentials were depolarized in the post-decompression phase following a 20-MPa high-pressure treatment; this could be explained by an increase in the Na^+- over K^+-permeability ratio and in intracellular $[Na^+]_i$. Following a 10-MPa high-pressure treatment, I_{Na}, I_K and I_{Ca} amplitudes were similar compared to controls but were significantly reduced by 25 to 35% after a 3-hr 20-MPa high-pressure treatment. Interestingly, the voltage-dependent inactivation of I_{Na} and I_{Ca} seemed to be more stable at high pressures compared to the activation parameters, as no significant changes were found up to a 20-MPa treatment. For higher pressure applications (e.g., 25 MPa), there seemed to be a marked loss of membrane integrity and I_{Na}, I_K and I_{Ca} almost disappeared.

Correspondence to: R.H.A. Fink; email: rainer.fink@urz.uni-heidelberg.de

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Introduction

High pressures up to 40 MPa have been applied to several different cellular preparations to characterize pressure-induced alterations of electrical membrane properties. For example, in bovine chromaffine cells, Macdonald (1997) found an increased open-probability of BK channels during high-pressure treatment, whereas calcium channels showed no signs of pressure-induced alterations for pressure treatments up to 40 MPa (Heinemann et al., 1987). In the same preparation, Heinemann and Conti (1992) described a reduction of functional sodium channels that was only partially reversible upon decompression. Furthermore, in squid giant axon (Conti et al., 1982) and Helix neurones (Harper, Macdonald & Wann, 1981) a reduction of potassium current amplitudes during high-pressure treatment was found.

Interestingly, high-pressure effects on cellular muscle preparations have been mainly investigated at the level of the contractile proteins (Ranatunga & Geeves, 1991; Vawda, Ranatunga & Geeves, 1995, 1996) and very little or even nothing is known about their sarcolemmal membrane conductances when exposed to high pressures. Such membrane conductance measurements with microelectrodes are particularly difficult to carry out in intact mammalian muscle cells due to their cellular geometry, their cable properties and their contractile response with depolarization. Recently, a modified two-microelectrode-voltage-clamp technique (2-MVC) was successfully applied to enzymatically isolated mammalian toe muscle fibers of the mouse to faithfully record membrane ion conductances (Friedrich, Ehmer &

Fink, 1999). As the isolated muscle cells are very fragile, it was not possible with our modified 2-MVC to measure ionic currents directly *during* a high-pressure application, but we were able to use this method to record membrane ion currents and potentials during the post-decompression phase following high-pressure treatments to 10, 20, 22.5 or 25 MPa at +4°C for 3 hr in adult single murine skeletal muscle fibers. Following a high-pressure treatment up to 20 MPa, the changes in membrane conductances for Na^+, K^+ and Ca^{2+} ions were only gradual and had reached a steady-state during the post-decompression phase. For higher pressures, electrical responses of single fibers were found to be markedly reduced. Therefore, our study provides new information on the partial or full recovery of ion conductances from high-pressure treatments up to 20 MPa and of specific alterations identified during the post-decompression phase. Further, we found that pressure applications higher than 20 MPa caused a marked functional decline or even a completely irreversible loss of membrane potentials and ion conductances in single skeletal muscle fibers.

These findings may provide a first insight into molecular susceptibilities of ion channels in native membranes of intact mammalian skeletal muscle cells to which high pressures have been applied. They may also be essential for the development of new technological applications based, e.g., on the adaptational mechanisms of deep-sea organisms to ambient pressures as high as 100 MPa (Macdonald, 2001). The present study may also be important to show limits for high-pressure cryopreservation of muscle tissue for transplantation in human beings (Kress et al., 1999).

Preliminary results of this work have been presented to the Biophysical Society USA (Friedrich et al., 2000) and at the International Conference on High Pressure Bioscience and Biotechnology (Kress et al., 1999).

Materials and Methods

Preparation

Twelve- to fourteen-week old adult male BALB/c mice were killed by a 10-minute exposure to a CO_2-atmosphere. All experiments were carried out according to the guidelines laid down by the local Animal Care Committee. *Mm. interossei* were dissected in Ringer's solution (*A*, *see* below) and transferred into Eppendorff caps. Before the high-pressure treatment, the preparation was precooled to +4°C in a refrigerator. After the high-pressure application, single fibers were enzymatically separated by collagenase treatment in Ringer's solution as previously described (Friedrich et al., 1999). This caused a roughly 45 min delay to the onset of the electrophysiological experiments. Fiber dimensions were on average similar before and after high-pressure treatment. From 400 control fibers the optically measured length was 601 ± 67 μm and diameter, 51 ± 10 μm (± SD).

Solutions

For electrophysiological experiments the following solutions were used: (*A*) isotonic Ringer's solution for recordings of fast sodium, (I_{Na}) and potassium (I_K) currents, and resting potentials E_R contained (mM): NaCl 136, KCl 4, $CaCl_2$ 2, Mg-acetate 1 and HEPES 10. For recordings of I_{Na} voltage-dependent inactivation, 6 mM TEA^+ and 5 mM 4-amino-pyridine (4-AP) were added to the solution to abolish I_K. (*B*) 10 mM free Ca^{2+}-containing hypertonic test solution for recordings of slow L-type calcium currents (I_{Ca}) contained (mM): Ca-acetate 10, Mg-acetate 1, TEA-Br 146, HEPES 10, CsBr 5, 4-AP 5, 3, 4-di-aminopyridine 5 and sucrose 300. The ionic strength ($\Gamma/2$ values) of solution (*B*) was calculated to 184 mM and had an osmolarity of 710 mosm l^{-1}. (*C*) hypertonic 'Cl^--free' solutions (chloride was completely replaced by equivalent amounts of acetate) with increasing concentrations of potassium of 4, 10, 20, 50, 80 and 130 mM were used to record membrane potentials. Sodium was added in equivalent decreasing amounts in order to keep the ionic strength ($\Gamma/2$ = 149 mM) and ionic equivalents (146 mM) constant (Fink, Stephenson & Williams, 1986). 1 mM KCl was added to all solutions to avoid junction potentials. The osmolarity of the 300 mM sucrose-containing solutions was about 650 mosmol l^{-1}. pH was adjusted to 7.4 using acetate acid.

High-Pressure Application

For the application of high pressure a modified commercial autoclave (PN 2500, Wepuko Hydraulics, Metzingen, Germany) was used. The effective chamber volume was 4 cm^3. The closed system was filled with destilled water. Pressure could be manually increased or decreased by a spindle screw (Wepuko Hydraulics) connected to a hand wheel (Kress et al., 2001). Compressing the water volume inside the autoclave by 0.053% resulted in a pressure increase of 10 MPa. Chamber pressure was measured by a high-pressure sensor (type 8219R, Burster Präzisionsmeßtechnik, Gernsbach, Germany). The temperature in the chamber was controlled with a digital thermoelement (type PDA.-48m/, Juchheim GmbH, Fulda, Germany) and kept constant by external perfusion with a water-ethanol mixture via a home-built cooling pump. Thereby, the chamber was also precooled to +4°C at atmospheric pressure before high-pressure applications. The hydrostatic pressure was increased using a standardized procedure: an increase of 0.1 MPa every 30 sec up to a value of 6 MPa, followed by an increase of 0.3 MPa every 30 sec up to the maximum pressure of 10, 20, 22.5 or 25 MPa. The maximum pressure was kept constant for 3 hr at 4°C. For the decompression the reverse procedure was used. This protocol produced a maximum number of functional fibers. This was qualitatively estimated by their response to a brief extracellular stimulation in the post-decompression state. Immediately after the high-pressure application *interossei* single muscle fibers were enzymatically separated as described before (Friedrich et al., 1999). Electrophysiological measurements on intact single fibers started approximately 45 min after the end of the decompression due to the time needed for the enzymatic separation. The measurements covered a period of four to five hours and no significant difference was found when comparing later experiments to those at earlier onset, indicating steady-state plateaus of post-decompression recoveries up to 20 MPa. For the control measurements, fibers were kept for similar time spans at atmospheric pressure at +4°C in order to rule out a 'rundown' of the measured properties due to the storage procedure.

To test for an unspecific pressure-dependent damage to the membrane with subsequent loss of intracellular enzymes we measured the creatine phosphokinase (CPK) enzyme activities in the incubation solution of some muscles following the high-pressure

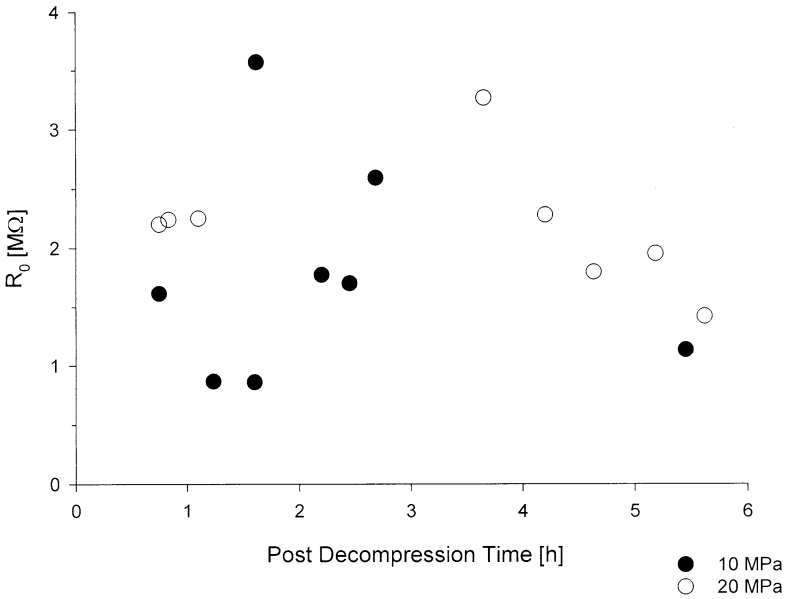

Fig. 1. Input Resistances R_0 from eight individual single fibers following a 3-hr 10 MPa (*filled circles*) and 20 MPa (*open circles*) high-pressure treatment measured during the post-decompression phase. There was no systematical rundown of the input resistance R_0, indicating a quasi-steady state during the post-decompression phase of up to six hours after the end of the decompression.

treatment. The CPK-levels obtained from the pressure-treated muscles were correlated to the CPK levels obtained from the corresponding muscles of the contralateral limbs, which were always kept under control conditions (i.e., atmospheric pressure).

Electrophysiology

Resting potentials were measured with micropipettes filled with 3 M KCl connected to a GeneClamp 500 amplifier (Axon Instr., Foster City, CA). It is common that some enzymatically isolated fibers show reduced resting potentials even under physiological control conditions. Therefore, for the statistical analysis we decided to evaluate only the eight highest resting potentials measured under control and test conditions. Membrane currents were recorded using the two-microelectrode-voltage-clamp technique (2-MVC) as previously described (Friedrich et al., 1999). To eliminate linear currents a P/4 linear leak subtraction method was used. The electrode separation distance was about 10 to 20 μm when inserting the electrodes opposite to each other in the fiber middle. High pressure-treated fibers were screened for changes in fast inward Na^+ and outward K^+ currents under isotonic conditions (I_{Na} and I_K) in the post-decompression phase. To suppress contractile movement during the rather long-lasting maintained depolarizations, recordings of calcium currents (I_{Ca}) were carried out under hypertonic conditions (e.g., Donaldson and Beam, 1983; Adrian, Chandler & Hodgkin, 1970).

Furthermore, considering the cable properties under isotonic conditions given by Friedrich et al. (1999), we determined the space constant λ of control fibers under hypertonic conditions to justify the application of the 2-MVC method under all conditions of our study. λ was measured by applying a -5 or -10 nA current step pulse of about 2-sec duration to the fiber and measuring the steady-state membrane potential differences ΔV for varying electrode separation distances Δx (10 to 350 μm). The current electrode was kept in the fiber middle while the potential-recording electrode was re-inserted at various distances. During this procedure, some damage to the membrane may occur in some fibers. To exclude a 'rundown' of the preparation during the recording of λ, the potential-recording electrode was withdrawn and re-inserted starting near the end of the fiber and approaching towards the middle. Experiments, in which ΔV decreased during this protocol, were discarded from the analysis. ΔV was plotted against Δx and could be well fitted by a cos*h*-function as expected from linear cable analysis, treating the fiber as a 'short cable' (*see* e.g., Weidmann, 1952). λ was obtained using a least-squares fit algorithm. In eight control fibers λ was 585 ± 197 μm.

The specific membrane capacitance C_m was measured as follows: potential steps in $+5$-mV increments from a holding potential of 0 mV were applied for 80 msec and the membrane current I_m was recorded. A 3-dB Bessel filter of 2 to 5 kHz was used. The depolarized holding potential of 0 mV was used to ensure that only passive membrane properties contributed to I_m. Slowly activating calcium currents as well as the gating-charge movement (e.g., Rios & Pizarro, 1991) will be inactivated at $E_h = 0$ mV, while other ionic conductances were blocked. The capacitive component I_C of I_m was integrated and the charge Q_C plotted against the membrane potential V. The input capacitance C_0 was calculated from the slope of the linear regression and divided by the optically measured surface area to yield C_m. It should be noted that in skeletal muscle the major part of the membrane capacitance is located in the T-system (reflected in the high C_m value of 4.7 ± 1.9 μF/cm^2 in $n = 15$ control fibers). Therefore, all currents are given as input currents I_0 in nA and were not normalized to fiber capacitance.

The input resistance R_0, taken as one indicator for electrophysiological fiber integrity, was determined as follows: from a holding potential of -80 mV, membrane currents I_m were recorded from up to 50 mV depolarizing and hyperpolarizing potential steps of 80-to 200-msec durations. The steady-state *I-V* relation, from which R_0 was calculated, was linear over the whole potential range tested. Therefore, R_0 in both the untreated control fibers as well as in the high pressure-treated fibers could be calculated by applying Ohm's law, i.e., by dividing the difference of the holding potential E_h and the measured resting potentials E_R by the holding current I_h (*see also* Harper et al., 1981). From the evaluation of R_0 in the post-decompression phase it is important to note that the fibers seemed to be in a quite stable steady state during the time span of up to 6 hr while the electrophysiological experiments were performed. As an example, Fig. 1 shows the input resistances R_0 from eight individual single fibers during the post-decompression phase following a 3-hr 10 MPa (*filled circles*) and 20 MPa (*open circles*) treatment each. It can be seen that there was no systematical 'rundown' of R_0 (or of the resting potentials E_R, *not shown*) during the experimental time span.

Data Acquisition and Preparation

Data were acquired using AxoTape 2.0 and pClamp6 software (Axon Instruments) and were analyzed using SigmaPlot 4 (Jandel Scientifics, USA). Standard deviations are given as ±SD with number n of observations. Data were fitted using the least-squares fit algorithms of Sigma Plot software. Significance was assessed using Student's t-test at the $P < 0.05$ level.

Results

Viability of Single Fibers Following High-Pressure Application and Cooling

The fibers' viability following the combined high-pressure and cooling treatment was qualitatively tested. Fibers were extracellularly stimulated and a contractile response was observed. In preliminary experiments, temperature during a 3-hr 20-MPa treatment was varied from +1°C to +7°C. For both, temperatures lower and higher than 4°C, fibers had a swollen morphological appearance, a loss of transparency and did not contract upon stimulation. However, even at a temperature of 4°C following a high-pressure treatment of 22.5 MPa and 25 MPa, most fibers did not survive the long-term pressure application. Except for two fibers it was impossible to perform two-micro-electrode measurements following a 25-MPa treatment, as all fibers, were destroyed by insertion of the second electrode. However, it was still possible to record resting potentials E_R from a few fibers with only one microelectrode inserted (*see* below).

Membrane Potential, Input Resistance and CPK Release from Control and High-Pressure-Treated Fibers

The calculated input resistances R_0 (*see* Methods) were (±SD): 1.5 ± 0.6 MΩ in $n = 46$ fibers at atmospheric pressure, 1.8 ± 0.8 MΩ in $n = 9$ fibers following a 3-hr 10-MPa high-pressure treatment and 2.7 ± 0.6 MΩ in $n = 14$ fibers following a 3-hr 20-MPa high-pressure treatment ($P < 0.001$ compared to controls). Following a 3-hr 22.5-MPa high-pressure treatment, R_0 seemed to decrease again (2.3 ± 0.4 MΩ in two fibers). It is important to note that the post-decompression phase can be considered as a new quasi-steady state during the experimental period from one up to five hours after decompression (*see* Methods, Fig. 1).

The resting membrane potentials E_R in the post-decompression phase following 10- to 25-MPa applications were recorded at atmospheric pressure in isotonic Ringer's solution. E_R in control fibers was measured after an equivalent period of time at +4°C and atmospheric pressure. The resting potentials evaluated for control fibers were -58.0 ± 7.5 mV (±SD, $n = 8$). After a 3-hr 10- and 20-MPa treatment, E_R was significantly smaller (-44.0 ± 7.7 mV and -42.0 ± 6.5 mV, $n = 8$, $P < 0.004$). Following a 3-hr 22.5- and 25-MPa treatment, E_R was markedly reduced to -6.1 ± 1.1 mV and -20.6 ± 4.9 mV ($P < 0.001$, $n = 8$). It should be noted that we did not observe a systematic rundown of E_R during the recording periods lasting up to six hours in the post-decompression phase.

To test if the decline in E_R may have been caused by an irreversible loss of intracellular potassium ions after treatment with increasing pressures, we recorded the membrane potential E_m with varying concentrations of extracellular potassium K_o over a range from 4 mM to 130 mM in four control fibers and in six fibers following a 3-hr 20-MPa treatment. The hypertonic K^+ solutions contained 300 mM sucrose to prevent K^+-contractures following exposure to high K_o (Dulhunty, 1977). E_m in 4 mM K^+-containing hypertonic solution measured -80.6 ± 2.8 mV in control fibers and -60.5 ± 9.4 mV following a 3-hr 20-MPa pressure application ($P < 0.003$). Compared to E_R in isotonic solution, E_m was markedly shifted towards more negative values when chloride was replaced by acetate. This finding is consistent with other mammalian skeletal muscle preparations (Dulhunty, 1977). In chloride-free solutions E_m is shifted towards more negative values because concentrations of chloride are not in equilibrium with the resting potential. The rate of change of E_m after an increase of K_o is more than 10 times slower in some mammalian muscle fibers. This is probably caused by the restricted diffusion into the t-tubules (Dulhunty, 1977). Thus, in 'Cl^--free' solution, E_m should mainly depend on the $[K^+]$ and $[Na^+]$ gradient and the specific ion permeability P_K or P_{Na} (Hodgkin & Horowicz, 1959). The intracellular potassium concentrations $[K]_i$ in control compared to those of 3-hr 20-MPa-treated fibers were estimated by extrapolation of the Goldman-Hodgkin-Katz equation (GHK) to the data (e.g., Hodgkin & Horowicz, 1959; Almers et al., 1984)

$$E_m = 59 \cdot \log \frac{[K]_o + \alpha[Na]_o}{[K]_i + \alpha[Na]_i} \ [\text{mV}] \qquad (1)$$

where $\alpha = P_{Na}/P_K$ denotes the relative permeability coefficient of the resting membrane and $[Na^+]_i$ was assumed to be ~19 mM in hypertonic solution. Fig. 2 shows the mean E_m values (±SD) and the least-squares fit to Eq. 1 for four control (*filled circles*, *solid line*) and six 3-hr 20-MPa-treated fibers (*open circles*, *dashed line*, $r^2 > 0.995$ for both plots). The values for $[K]_i$ from extrapolation of the fits were found to be slightly reduced in 20-MPa-treated fibers (237 mM) compared to controls (247 mM). The relative permeability coefficient α was 0.04 for control fibers. This

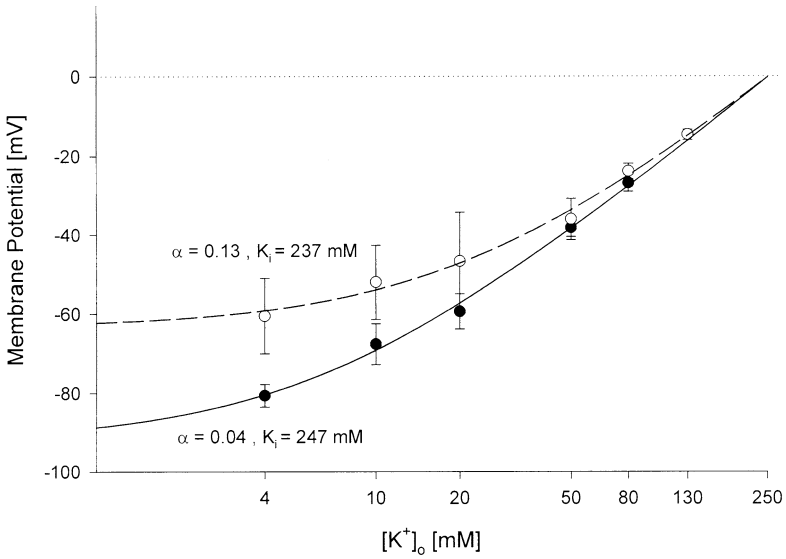

Fig. 2. Membrane potentials in single *interossei* fibers following a 3-hr high-pressure treatment at 4°C. Fibers were bathed in hypertonic solution with varying extracellular potassium concentration $[K^+]_o$. Membrane potentials are shown for $n = 4$ control fibers (*filled circles*) and $n = 5$ fibers following a 3-hr 20-MPa treatment (*open circles*, mean ± SD). Data were fitted by the Goldmann-Hodgkin-Katz equation (Eq. 1; control: *solid line*, 20 MPa: *dashed line*), $\alpha = P_{Na}/P_K$ was 0.04 in control fibers and increased about threefold to 0.13 after the 20-MPa high-pressure treatment. The extrapolated intracellular potassium concentration $[K^+]_i$ was only slightly reduced in 20 MPa-treated fibers (237 mM vs. 247 mM in controls).

value is similar to that reported for rat muscle ($\alpha = 0.05$, Pappone, 1980). Interestingly, following the 3-hr 20-MPa treatment, α increased about threefold to 0.13 in our preparation. Thus, it seems unlikely that the observed depolarization of E_R seen in isotonic Ringer's solution following a 3-hr 20-MPa high-pressure treatment is mainly due to a loss of intracellular potassium ions but may rather be explained by a reduced ability of the resting membrane to discriminate sodium from potassium ions. Furthermore, we quantified the CPK levels in the Ringer's solution the muscles were incubated in during the prolonged high-pressure treatment and compared them to the levels obtained from the contralateral toe muscles of the same animal kept under control conditions. An increase of CPK enzyme activity in the extracellular medium generally indicates unspecific membrane damage with a leakage of intracellular proteins. Following a 3-hr 20-MPa treatment, CPK ratios R_{CPK} (pressure treatment *vs.* control) in five *interossei* muscle pairs were 1.42 ± 0.47. To account for a pressure-induced change to the enzyme activity itself we also evaluated R_{CPK} for high pressure-treated CPK in Ringer's solution *vs.* CPK in solution kept under control conditions. R_{CPK} following a 20-MPa treatment was 0.83 ± 0.02 ($n = 5$), indicating only a minor inactivation of the enzyme activity itself by high pressure. Following a 25-MPa treatment, R_{CPK} was 0.99 for one *interossei* pair and 0.93 ± 0.06 for CPK activity in Ringer's solution ($n = 2$). During dissection of *interossei* muscles there is always some damage to the preparation. This might result in an increase of CPK that is not pressure-induced. To overcome this problem, we also measured R_{CPK} for the *EDL* (*extensor digitorium longus*) muscle in the same animals. This muscle can be more atraumatically resected. R_{CPK} was 0.91 ± 0.17 ($n = 9$, 20 MPa) and 1.18 ± 0.03 ($n = 5$, 25 MPa). From these data it is apparent that a major unspecific damage to the membrane and a loss of intracellular proteins can be excluded even for the 25-MPa treatment.

Fast Sodium and Potassium Membrane Currents (I_{Na}, I_K) in the Post-Decompression Phase

In Fig. 3*A* voltage-clamp recordings of the inward I_{Na} and the delayed outward I_K are shown for a single fiber in isotonic Ringer's solution following a 20-MPa treatment. The holding potential was −90 mV and currents were measured with 10-mV voltage increments up to +40 mV. Fig. 3*B* shows the evaluated maximum peak I_{Na} amplitudes ($\hat{I}_{Na}$) for control fibers ($n = 26$) and following a 3-hr high-pressure application to 10 MPa ($n = 5$), 20 MPa ($n = 18$), and 25 MPa ($n = 2$). Following a 3-hr 10 MPa pressure application, $\hat{I}_{Na}$ was on average only slightly smaller (-751 ± 196 nA, ± SD, ± 88 nA, ± SEM) than in control fibers (-788 ± 164 nA, ± 32 nA). However, following a 3-hr 20-MPa application $\hat{I}_{Na}$ was significantly reduced (-585 ± 201 nA, ± 48 nA, $P < 0.001$) compared to controls. For higher pressures (e.g., 25 MPa) I_{Na} almost disappeared in our preparations. The apparent reversal potentials E_{Na} measured $+57.8 \pm 19.1$ mV in control fibers and were very similar following a 3-hr 10-MPa high-pressure application (+55.5 ± 10.4 mV). However, following a 3-hr 20-MPa high-pressure treatment, E_{Na} was shifted to less positive values compared to the controls ($+37.5 \pm 14$ mV, $P = 0.01$).

In Fig. 3*C* the effect of high-pressure application on the steady-state amplitudes of potassium currents $I_{K,ss}$ is shown for a membrane potential of +40 mV. Following a 3-hr 10-MPa application, $I_{K,ss}$ in six fibers measured 356 ± 110 nA (± SD, ± 45 nA, ± SEM)

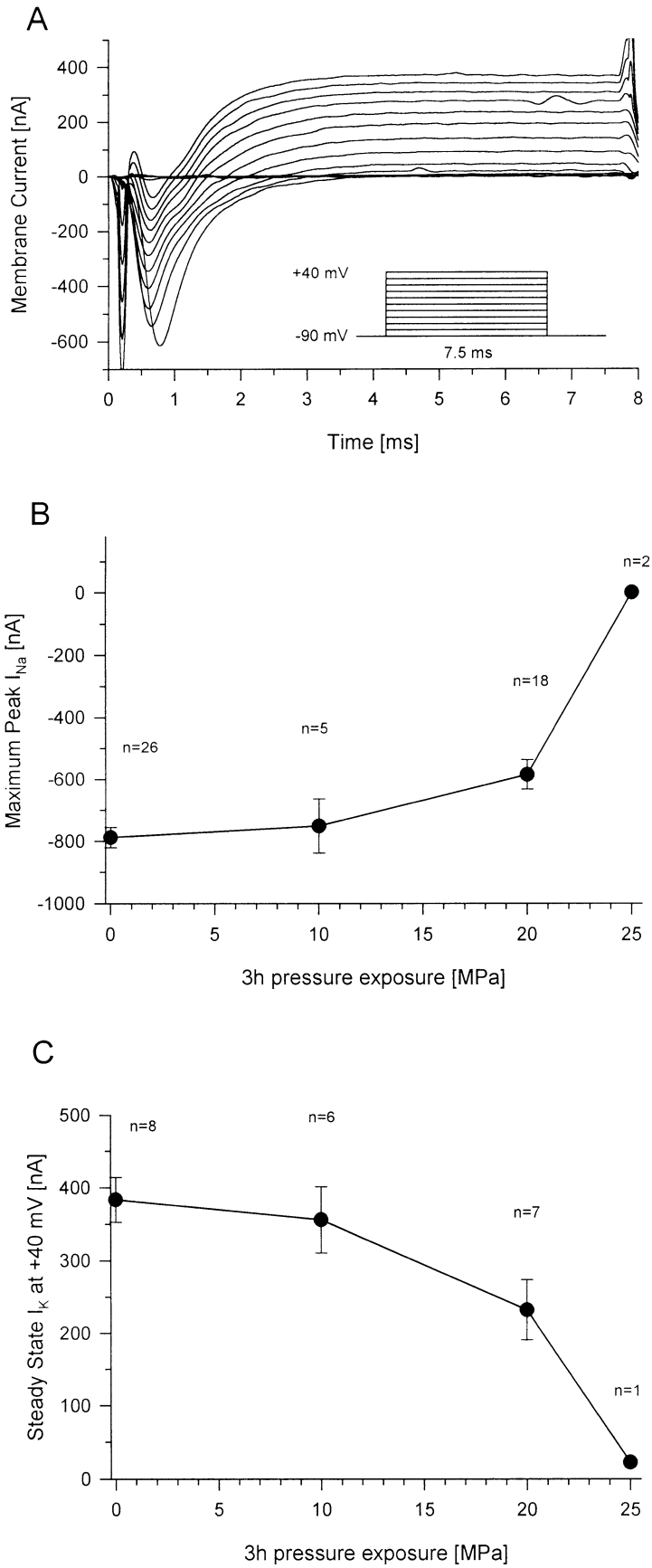

Fig. 3. Fast I_{Na} and I_K in single *interossei* fibers after a 3-hr high-pressure treatment at 4°C. (*A*) Membrane currents in isotonic Ringer's solution were elicited from a holding potential of −90 mV with step pulse protocols shown in the inset for a fiber following a 20-MPa treatment. I_{Na} and I_K could be identified using TTX, TEA^+ and 4-AP (*see*, e.g., Fig. 5). (*B*) shows the pressure-dependent maximum peak current amplitudes for I_{Na} following a 3-hr high-pressure application to the values indicated. Note that following pressures higher than 20 MPa, I_{Na} virtually disappeared. (*C*) Steady-state I_K amplitudes for a selected membrane potential of +40 mV after the 3-hr pressure application as indicated. Following 25 MPa applications, only a very small I_K component remained. Error bars in (*B*) and (*C*) are SEM.

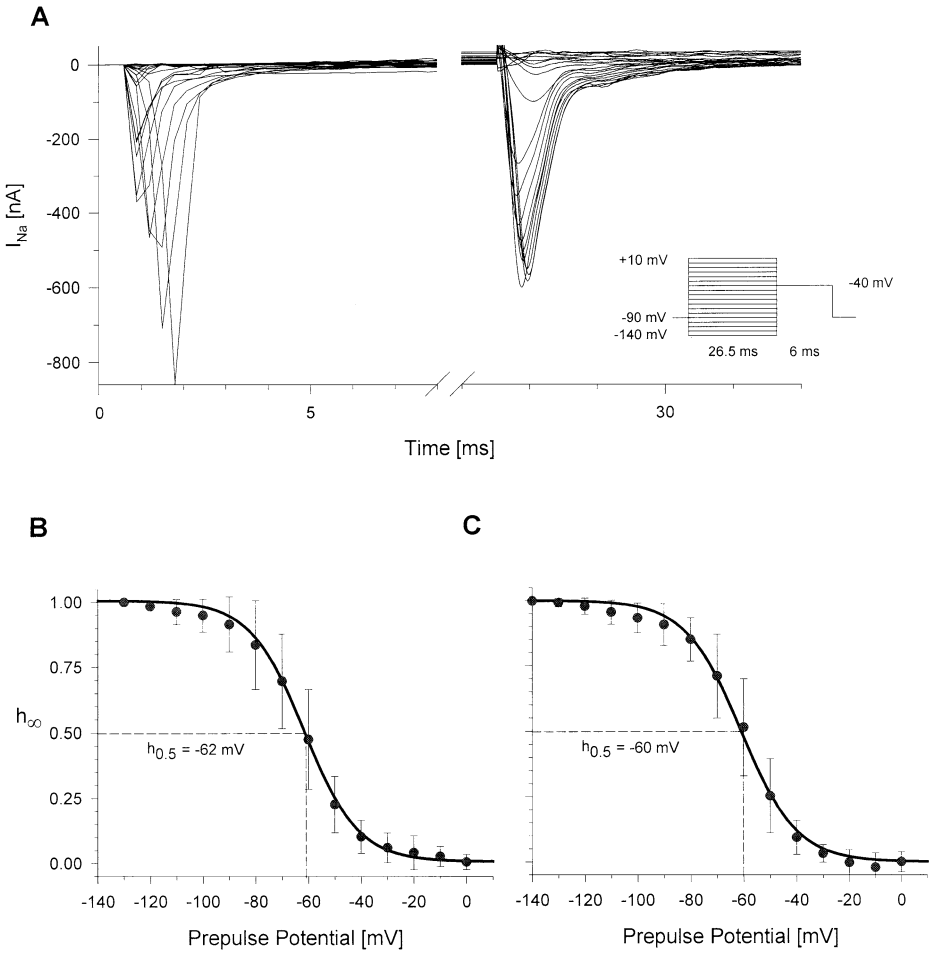

Fig. 4. Voltage-dependent inactivation (h_∞) of I_{Na}, in single *interossei* fibers after a 3-hr 20-MPa pressure exposure at 4°C. (*A*) Recordings from a single fiber following a 20-MPa treatment. The double-pulse protocol with a test pulse to −40 mV and variable prepulses is shown in the inset. I_K was suppressed by adding 6 mM TEA^+ and 5 mM 4-AP to the isotonic Ringer's solution. With increasing prepulse potentials, peak amplitudes during the test pulse decreased. The voltage-dependent inactivation h_∞ was determined as described, e.g., by Almers et al. (1984). The results are shown in (*B*) for $n = 11$ fibers following a 20-MPa application (*filled circles*, ±SD) and in (*C*) for eight control fibers. The solid lines are Boltzmann fits calculated from the mean half-inactivation potential and slope factors of all individual fibers. $h_{0.5}$ was -62 ± 6 mV following a 20 MPa treatment and was similar to control fibers (-60 ± 5 mV).

and was similar to $I_{K,ss}$ in eight control fibers (383 ± 85 nA, ± 30 nA). $I_{K,ss}$ was significantly reduced following a 3-hr 20-MPa high-pressure treatment (232 ± 110 nA, ± 41 nA, $n = 7$, $P < 0.01$). Following a 3-hr pressure treatment at 25 MPa only a very small $I_{K,ss}$ component less than 5% of controls was found under our experimental conditions.

To further specify the molecular sites of high-pressure action on the sodium channels we investigated the voltage-dependent inactivation h_∞ of I_{Na}. The reduction of the maximum peak I_{Na} amplitude for a fixed membrane-potential step from −90 mV to −40 mV was studied for varying prepulse amplitudes. Inactivation h_∞ was then evaluated as described, e.g., by Almers et al. (1984). Figure 4*A* shows a representative example of such double-pulse recordings obtained from one single fiber following a 3-hr 20-MPa treatment. The external isotonic Ringer's solution contained 6 mM TEA^+ and 5 mM 4-AP to block outward I_K. It was interesting to find that following a 3-hr 20-MPa application, h_∞, as shown in Fig. 4 *B*, had half inactivation values ($h_{0.5} = -62 \pm 6$ mV, $n = 11$) similar to control fibers ($h_{0.5} = -60 \pm 5$ mV, $n = 8$, Fig. 4*C*). Note, that the solid lines in (*B*) and (*C*) represent the mean Boltzmann-fit from the equation

$$h_\infty = \left\{1 + e^{\frac{V - h_{0.5}}{b}}\right\}^{-1} \quad (2)$$

where V denotes the prepulse potential, $h_{0.5}$ the half-inactivation potential and b the slope of the fit. The mean $h_{0.5}$ and b were obtained from the fits to the individual data traces.

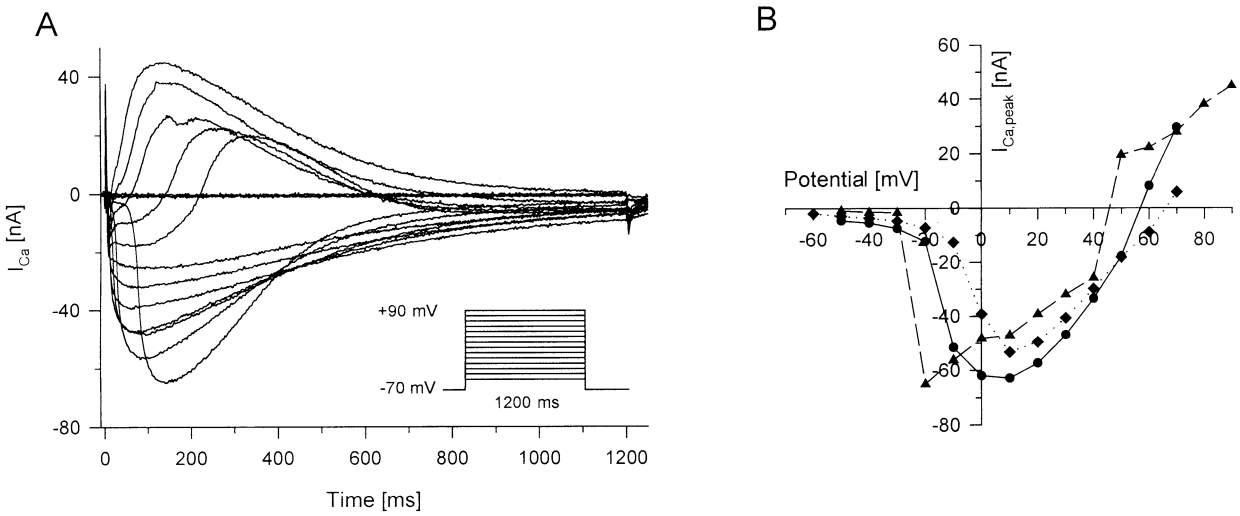

Fig. 5. Slowly activating L-type calcium currents (I_{Ca}) in single *interossei* fibers after a 3-hr high-pressure treatment at 4°C. (*A*) A set of I_{Ca} from a single fiber following a 10-MPa high-pressure treatment. The kinetics were similar to control fibers. Holding potential was −70 mV and pulse steps had durations of 1200 msec with 10-mV increments from –50 mV to +90 mV, as shown in the inset. (*B*) *I-V* plot from the fiber in (*A*) (*triangles*) together with the data from one control fiber (*circles*) and a 20-MPa-treated fiber (*diamonds*). Threshold potentials ranged from −30 mV to −20 mV. Reversal potentials were markedly increased following a 3-hr 20-MPa high-pressure treatment. Also, peak amplitudes were reduced by about 30%.

Slow L-Type Calcium Currents (I_{Ca}) in the Post-Decompression Phase

To test the reversibility of high pressure-induced effects on L-type Ca^{2+} channels (DHP-receptors), which are almost exclusively located in the transverse tubular system (TTS; Almers et al., 1981), the voltage-dependent Ca^{2+} current (I_{Ca}) was measured in isolated *interossei* fibers under hypertonic conditions. Fig. 5*A* shows I_{Ca} recordings from a representative single fiber following a 3-hr 10-MPa high-pressure application. The holding potential was −70 mV and the 1.2-sec long pulses ranged from −50 mV to +90 mV in 10-mV increments. Fig. 5*B* shows the *I-V*-relations for the fiber shown in (*A*) (10 MPa, *triangles*), as well as for a representative control fiber (*circles*) and a fiber following a 3-hr 20-MPa application (*diamonds*). Following a 10-MPa treatment I_{Ca} was similar to control fibers regarding activation kinetics but had slightly smaller maximum peak amplitudes ($I_{Ca,\,max}$) of -62 ± 23 nA ($n = 9$) compared to control fibers (-72 ± 17 nA, $n = 15$). The apparent reversal potentials E_{Ca} were also similar ($+47.8 \pm 4.6$ mV, $n = 5$ control fibers and $+46.5 \pm 0.2$ mV, $n = 2$ fibers following 10-MPa treatment, respectively). For 20 MPa-treated fibers $I_{Ca,max}$ was reduced by roughly 30% compared to control fibers (-50 ± 6 nA, $n = 10$, similar fibers dimensions). Interestingly, E_{Ca} values were significantly increased to $+71 \pm 6$ mV in four fibers ($P < 0.01$). Following higher pressure applications (e.g., 22.5 MPa) experiments with two microelectrodes became extremely difficult and I_{Ca} could no longer be recorded. As the holding currents were not significantly different for all pressures (-31.3 ± 5.5 nA for $n = 16$ control fibers, -30.5 ± 14.4 nA for 10 MPa-, $n = 9$, -31 ± 13 for 20 MPa-, $n = 16$ and -27.6 ± 12.4 nA for 22.5 MPa-treated fibers, $n = 3$, $P > 0.38$), the failure to record I_{Ca} for higher pressures cannot simply be explained by a clamp instability but is more likely due to the pressure treatment.

The voltage-dependent inactivation of calcium channels F_∞ (Matsuda, Volk & Shibata, 1990; Friedrich et al., 1999) was recorded with a double-pulse protocol as shown for a single fiber following a 3-hr 20-MPa high-pressure application in Fig. 6*A*. 1.8-sec long prepulses of varying potential amplitudes ($E_h = -70$ mV) were followed by a 750-msec test pulse to 0 mV. In (*B*) and (*C*), F_∞ is shown for four single fibers following a 10-MPa exposure and five single fibers following a 20-MPa exposure. (*D*) shows F_∞ for $n = 10$ control fibers. The solid lines represent the mean Boltzmann fits from the equation

$$F_\infty = \left\{1 + e^{\frac{V - f_{0.5}}{b}}\right\}^{-1} \quad (3)$$

with half-inactivation potential $f_{0.5}$ and slope b. Note that the data shows some marked deviation from the mean Boltzmann fit, especially for hyperpolarizing prepulses; this can be due to contamination of I_{Ca} by calcium-ion depletion in the T-system and increasing non-linearities affecting the 'leak-subtraction' procedure as previously shown (Friedrich et al., 1999, 2001). Following a 10 MPa application, $f_{0.5}$ and b were -23.6 ± 4.7 mV and -11.5 ± 7.5 mV^{-1} and were similar to control fibers ($f_{0.5} = -23.4 \pm 6$ mV,

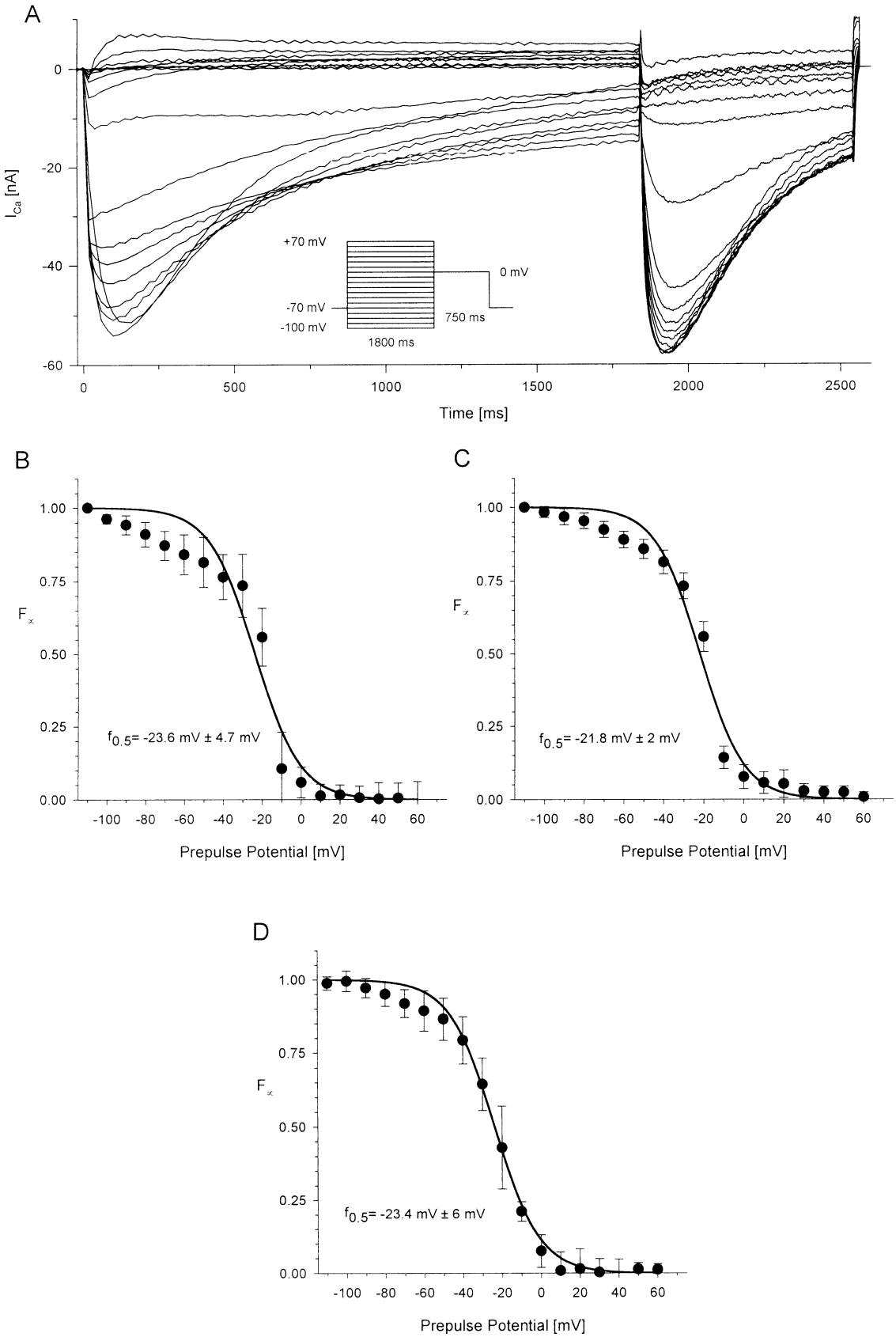

Fig. 6. Voltage-dependent inactivation (F_∞) in single *interossei* fibers after a 3-hr pressure exposure at 4°C. (*A*) I_{Ca} recorded in a single fiber following a 10-MPa pressure treatment. The double-pulse protocol with fixed test pulse (0 mV) and variable prepulses is shown in the inset. With increasing prepulse potentials, peak amplitudes during the test-pulse decreased. The calculated F_∞ is shown in (*B*) for $n = 4$ fibers after 10-MPa exposure, (*C*) for $n = 5$ fibers after 20-MPa exposure and (*D*) for $n = 10$ control fibers. The solid lines are Boltzmann fits. The mean half-inactivation potentials and slope factors were similar for controls and following the high-pressure applications tested.

$b = -8.9 \pm 3.9$ mV^{-1}). Following a 20-MPa high-pressure treatment, $f_{0.5}$ was -21.8 ± 2 mV and $b = -10.9 \pm 2$ mV^{-1} — again, both similar to control fibers.

Discussion

Input Resistance R_0 in the Post-Decompression Phase

We have chosen to follow the input resistance R_0 as an indicator of the electrical integrity of our single-fiber preparations during the steady-state post-decompression phase. As it seems unlikely that the internal resistivity should be changed by the high-pressure treatment, changes in R_0 qualitatively reflect changes in the specific membrane resistance (*see* e.g., Fink & Lüttgau, 1976). Interestingly, R_0 increased with pressure up to 20-MPa about 1.8-fold, similar to findings reported for bifurcating axons of lobster, where an increase of R_0 with pressure during the high-pressure application has been found (Grossman & Kendig, 1984*a*). However, following 22.5-MPa, a tendency for R_0 to decrease could be observed. As for higher pressures than 22.5 MPa, e.g., at 25 MPa, R_0 could not be measured anymore — we assume due to a deterioration of electrical membrane functioning, e.g., an increasing leak conductance, starting above 20 MPa. However, even for pressures as high as 25 MPa, a complete unspecific breakdown of the mechanical membrane integrity does not seem to occur, as judged from our CPK measurements.

E_R, I_{Na} and I_K in the Post-Decompression Phase

Following a high-pressure treatment up to 20 MPa, a depolarization of the resting potentials E_R was observed. This could be due to a loss of K^+ ions from the myoplasm, as, e.g., would be expected from a pressure-induced decrease of the Na/K-ATPase transport rate during high-pressure exposure (Goldinger et al., 1980*a*, *b*, human erythrocytes; Hall et al., 1982; Shelton and Macdonald, 1987; fish erythrocytes; Li et al., 1990, frog skin). Interestingly, Goldinger et al. (1980*a*) could show that the decrease of Na/K-ATPase transport rate was accompanied by an increase in ATPase activity and ATP consumption during high-pressure applications. In this context it should also be noted that the increase of partial gas pressures during pressurization, i.e., for oxygen, may have had direct or indirect effects on the cellular metabolism and membrane function, as oxygen sensors are known to be present in skeletal muscle (e.g., Kourie, 1997; Eu et al., 2000).

To clarify if a decrease in $[K^+]_i$ can explain the decrease of E_R in our preparations, we recorded the membrane potential at various extracellular K^+ concentrations $[K]_o$ in hypertonic solution. The data obtained for control fibers kept under atmospheric conditions were similar to those reported for rat *sternomastoid* fibers (Dulhunty, 1977). The calculated high intracellular potassium concentrations $[K]_i$ (Eq. 1) for control fibers (247 mM) can be explained by a shrinking of the muscle fibers in the hypertonic solution (e.g., Almers et al., 1984; Adrian, 1956) if one assumes a reduction in fiber volume by 20 to 30%. From our data, following a 3-hr 20-MPa treatment, $[K^+]_i$ was only slightly reduced (237 mM). The reduction in $[K^+]_i$ is expected to be accompanied by a corresponding increase of intracellular sodium $[Na^+]_i$ from about 10 mM (*see also* Adrian & Marshall, 1977: 16 mM under their hypertonic conditions) to 19 mM under isotonic conditions, as calculated from the approximately 20 mV reduction of the apparent reversal potentials E_{Na} of I_{Na} following a 3-hr 20-MPa high-pressure treatment. However, the reduction of $[K^+]_i$ itself can only partly account for the decrease of E_R seen under isotonic conditions. Instead, the application of the GHK-equation indicates a threefold increase in the P_{Na}/P_K ratio following a 3-hr 20-MPa high-pressure treatment.

Following a 3-hr 10-MPa pressure treatment I_{Na} and steady-state I_K showed similar amplitudes compared to control current amplitudes, whereas a significant decrease of both I_{Na} and I_K occurred following a 20-MPa treatment. Following a 10- and 20-MPa treatment, voltage-dependent inactivation for sodium currents was not altered. After pressure treatment of more than 20 MPa, voltage clamp with two microelectrodes became increasingly difficult and I_{Na} and I_K virtually disappeared following a 25-MPa treatment. Our results after prolonged high-pressure treatment up to 20 MPa in intact fibers for I_{Na} and I_K are in general agreement with reported pressure-induced channel alterations in other preparations. For example, the number of functional sodium channels of adrenal bovine chromaffin cells (Heinemann & Conti, 1992), large crustacean motor neuron (Grossman & Kendig, 1984*a*, *b*; Kendig & Grossman, 1986) and in amphibian myelinated nerve cells (Kendig, 1984*a*, *b*) was found to be reduced by high-pressure application. This effect not only resulted in reduced I_{Na} peak current amplitudes but was also only partially reversible upon decompression (Heinemann et al., 1987). For potassium currents the reported data are more controversial. Similar to the decline of I_K seen in our mammalian single-muscle fiber preparation Harper et al. (1981) found a pronounced decrease of delayed outward I_K in *Helix* neurons at 20.8 MPa, which was in contrast to our results of full reversibility upon decompression. For skeletal muscle, there is to our knowledge no systematic investigation of I_K under high pressure to date. Interestingly, in squid giant axon Conti et al.

(1982) found even an increase in the steady-state I_K components during a pressure application of 41 MPa.

I_{Ca} in the Post-Decompression Phase

The slowly activating calcium currents (I_{Ca}) showed only minor changes following a 10-MPa treatment, which became more prominent after a 20-MPa application in our muscle fiber preparations. For example, the shift in the apparent reversal potentials E_{Ca} of about 20 mV towards more positive potentials is a strong indication that following such high pressures the selectivity of calcium channels seems to increase. However, following pressures higher than 20 MPa, I_{Ca} could no longer be recorded, probably due to the increase in leak conductance and the fragility of the fibers. Similar to sodium channels, the voltage-dependent inactivation (F_∞) at 10 and 20 MPa was unaltered. It is very interesting that in contrast to our results on intact muscle fibers Heinemann et al. (1987) found no alterations of I_{Ca} in adrenal bovine chromaffin cells for pressures even up to 40 MPa.

In summary, our study provides new information about specific alterations of input resistances, membrane potentials, intracellular ion concentrations and voltage-gated ionic conductances in mammalian single toe-muscle fibers in the post-decompression phase following pressure applications up to 20 MPa. Surprisingly, in all voltage-gated channels investigated the voltage-dependent inactivation gating properties seemed to be more stable than the activation parameters. However, for higher pressure applications, there seems to be a marked irreversible damage to the membranes of mammalian skeletal muscle.

In conclusion, our findings support some specific pressure-induced alterations of membrane properties rather than an unspecific stressing of the heterogeneous membrane structures in skeletal muscle.

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References

Adrian, R.H. 1956. The effect of internal and external potassium concentration on the membrane potential of frog muscle. *J. Physiol.* **133:**631–658

Adrian, R.H., Marshall, M.W. 1977. Sodium currents in mammalian muscle. *J. Physiol.* **268:**223–250

Adrian, R.H., Chandler, W.K., Hodgkin, A.L. 1970. Voltage clamp experiments in striated muscle fibers. *J. Physiol.* **208:**607–644

Almers, W., Roberts, W.M., Ruff, R.L. 1984. Voltage clamp of rat and human skeletal muscle: measurements with an improved Loose-Patch technique. *J. Physiol.* **347:**751–768

Almers, W., Fink, R., Palade, P.T. 1981. Calcium depletion in frog muscle tubules: the decline of calcium current under maintained depolarization. *J. Physiol.* **312:**177–207

Conti, F., Fioravanti, R., Segal, J.R., Stuehmer, W. 1982. Pressure dependence of the sodium currents of squid giant axon. *J. Membrane Biol.* **69:**23–34

Donaldson, P.L., Beam, K.G. 1983. Calcium currents in a fast twitch skeletal muscle of the rat. *J. Gen. Physiol.* **82:**449–468

Dulhunty, A. 1977. K-contractures and membrane potential in mammalian skeletal muscle. *Nature* **266:**75–78

Eu, J.P., Sun, J., Xu, L., Stamler, J.S., Meissner, G. 2000. The skeletal muscle calcium release channel: coupled O_2 sensor and NO signalling functions. *Cell* **104:**499–509

Fink, R.H.A., Stephenson, D.G., Williams, D.A. 1986. Potassium and ionic strength effects on the isometric force of skinned twitch muscle fibers of the rat and toad. *J. Physiol.* **370:**317–337

Fink, R.H.A., Lüttgau, H.C. 1976. An evaluation of the membrane constants and the potassium conductance in metabolically exhausted muscle fibers. *J. Physiol.* **263:**215–238

Friedrich, O., Ehmer, Th., Uttenweiler, D., Vogel, M., Barry, P.H., Fink, R.H.A. 2001. Numerical analysis of Ca^{2+} depletion in the transverse tubular system of mammalian muscle. *Biophys. J.* **80:**2046–2055

Friedrich, O., Kress, K.R., Ludwig, H., Fink, R.H.A. 2000. Reversibility of high pressure-induced effects on ionic currents in single murine toe muscle fibers. *Biophys. J.* **78:**363A

Friedrich, O., Ehmer, T., Fink, R.H.A. 1999. Calcium currents during contraction and shortening in enzymatically isolated murine skeletal muscle fibers. *J. Physiol.* **517:**757–770

Goldinger, J.M., Kang, B.S., Choo, J.E., Paganelli, C.V., Hong, S.K. 1980*a*. Effect of hydrostatic pressure on ion transport and metabolism in human erythrocytes. *J. Appl. Physiol.* **49:**224–231

Goldinger, J.M., Kang, B.S., Morin, R.A., Paganelli, C.V., Hong, S.K. 1980*b*. Effect of hydrostatic pressure on active transport, metabolism and the Donnan equilibrium in human erythrocytes. *In:* Proceedings of the VIIth Symp. on Underwater Physiology. Bachrach, A.J., Matzen, M.M., editors. pp. 589–599. Undersea Med. Soc. Inc., Bethesda, MD

Grossman, Y., Kendig, J.J. 1984*a*. Pressure and temperature: time-dependent modulation of membrane properties in a bifurcating axon. *J. Neurophysiol.* **52**:692–708

Grossman, Y., Kendig, J.J. 1984*b*. Time dependent effects of pressure and temperature on an integrative axon. *In:* Underwater Physiology, Vol. 8. Bachrach, A.J., Matzen, M.M., editors. pp. 583–585. Undersea Med. Soc. Inc., Bethedsa, MD

Hall, A.C., Ellory, J.C., Klein, R.A. 1982. Pressure and temperature effects on human red cell cation transport. *J. Membrane Biol.* **68:**47–56

Harper, A.A., Macdonald, A.G., Wann, K.T. 1981. The action of hydrostatic pressure on the membrane currents of *helix* neurones *J. Physiol.* **311**:325–339

Heinemann, S.H., Conti, F. 1992. Non-Stationary noise analysis and its applications to patch clamp recordings. *In:* Methods in Enzymology, Vol. 207. Ion channels. Rudy, B., Iverson, L.E., editors. pp. 131–148. Academic Press, San Diego, CA

Heinemann, S.H., Conti, F., Stuehmer, W., Neher, E. 1987. Effects of hydrostatic pressure on membrane processes. Sodium channels, calcium channels and exocytosis. *J. Gen. Physiol.* **90:**765–778

Hodgkin, A.L., Horowicz, P. 1959. The influence of potassium and chloride ions on the membrane potential of single muscle fibers. *J. Physiol.* **148**:127–160

Kendig, J.J., Grossman, Y. 1986. Homogeneous and branching axons: differing responses to anaesthetics and pressure. *In:* Molecular and cellular mechanisms of anaesthetics. Roth, S.H., Miller, K.W., editors. pp. 333–353. Plenum, New York

Kendig, J.J. 1984*a*. Ionic currents in vertebrate myelinated nerve at hyperbaric pressure. *Amer. J. Physiol.* **246:**C84–C90

Kendig, J.J. 1984*b*. Nitrogen narcosis and pressure reversal of anaesthetic effects in node of Ranvier. *Amer. J. Physiol.* **246:**C91–C95

Kourie, J.I. 1997. A redox O_2 sensor modulates the SR Ca^{2+} countercurrent through voltage and Ca(2+)-dependent Cl-channels. *Amer. J. Physiol.* **272:**C324–332

Kress, K.R., Friedrich, O., Ludwig, H., Fink, R.H.A. 2001. Reversibility of high pressure effects on the contractility of skeletal muscle. *J. Muscle Res. Cell Motility* **22:**379–389

Kress, K.R., Friedrich O., Ludwig, H., Fink, R.H.A. 1999. Reversibility of high pressure effects in isolated adult murine skeletal muscle fibers tested at pressures up to 600bar and a temperature of +4°C. *In:* Advances in high pressure Bioscience and Biotechnology, Proceedings of the International Conference on High Pressure Bioscience and Biotechnology, Heidelberg 30th August–3rd September 1998. pp. 551–544. Springer Berlin, Heidelberg, New York

Li, M., Hong, S.K., Goldinger, J.M., Duffey, M.E. 1990. Interaction of heptanol and pressure on sodium and chloride transport by frog skin. *J. Appl. Physiol.* **69:**1883–1892

Macdonald, A.G. 2001. Effects of high pressure on cellular processes. *In:* Cell Physiology Sourcebook: A Molecular Approach, 3rd ed., Sperelakis, N., editor. pp. 1003–1023. Academic Press

Macdonald, A.G. 1997. Effect of high hydrostatic pressure on the BK channel in bovine chromaffin cells. *Biophys. J.* **73:**1866–1873

Matsuda, J.J., Volk, K.A., Shibata, E.F. 1990. Calcium currents in isolated rabbit coronary arterial smooth muscle myocytes. *J. Physiol.* **427:**657–680

Pappone, P.A. 1980. Voltage-clamp experiments in normal and denervated mammalian skeletal muscle fibers. *J. Physiol.* **306:**377–410

Ranatunga, K.W., Geeves, M.A. 1991. Changes produced by increased hydrostatic pressure in isometric contractions of rat fast muscle. *J. Physiol.* **441:**423–431

Rios, E., Pizarro, G. 1991. Voltage sensor of excitation-contraction coupling in skeletal muscle. *Physiol. Rev.* 71:849–908

Shelton, C.J., Macdonald, A.G. 1987. Effect of hydrostatic pressure on $^{86}Rb^+$ influx in the erythrocyte of the plaice. *Comp. Biochem. Physiol.* **88A:**481–485

Vawda, F., Ranatunga, K.W., Geeves, M.A. 1995. Pressure-induced changes in the isometric contractions of single intact muscle fibers at low temperatures. *J. Muscle Res. Cell Motility* **16:**412–419

Vawda, F., Ranatunga, K.W., Geeves, M.A. 1996. Effects of hydrostatic pressure on fatiguing frog muscle fibers. *J. Muscle Res. Cell Motility* **17:**631–636

Weidmann, S. 1952. The electrical constants of Purkinje fibers. *J. Physiol.* **118:**348–360