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ORIGINAL COMMUNICATION

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Critical illness myopathy serum fractions affect membrane excitability and intracellular calcium release in mammalian skeletal muscle

Abstract The pathogenesis of myopathies occurring in critically ill patients (*critical illness myopathy*, CIM) is poorly understood. Both local and systemic responses to sepsis and other severe insults to the body are presumed to be involved but the precise mechanisms by which muscle function is impaired are far from clear. To elucidate such mechanisms we investigated the effects of blood serum fractions (5 kDa to 100 kDa molecular weight cut-off, MWCO) from patients with CIM and from control persons on membrane and contractile functions in intact mammalian single skeletal muscle fibres and chemically skinned fibre bundles. In intact fibres, resting membrane potentials were less negative when exposed to CIM serum fractions compared with control serum fractions. Half-width and maximum rise time of action potentials (AP) were smaller in CIM serum low MWCO fractions vs. control serum. Peak amplitudes of fast inward sodium currents (I_{Na}) were increased by low MWCO-CIM fractions compared with control sera fractions. Additionally, voltage dependent inactivation of I_{Na} was shifted towards more positive potentials by high MWCO fractions of CIM sera. In skinned fibres, pCa-force relations were similar in CIM and control serum fractions but peak force of Ca^{2+} induced force transients was decreased by low MWCO-CIM vs. control serum fractions. Our results (i) provide the first evidence that serum from CIM patients affects membrane excitability and the excitation-contraction coupling process at the level of the sarcoplasmic reticulum Ca^{2+} release of mammalian muscle fibres and (ii) also show that even control serum fractions 'per se' alter the response to important physiological membrane and contractility parameters compared with physiological saline.

Key words skeletal muscle · critical illness myopathy · membrane excitability · contractility · calcium regulation

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Introduction

Myopathy and polyneuropathy occur in critically ill patients during treatment on the intensive care unit causing generalized muscle weakness, failure of weaning and prolonged rehabilitation [1–4]. These intensive care 'de novo' pathologies can affect up to 80 % of patients intubated for more than two weeks [5–9]. They also highly increase the risks for secondary complications such as pneumonia, deep vein thrombosis and pulmonary embolism. It is not clear whether these entities have a common or separate origin. Muscle biopsies from intensively treated patients revealed three morphologically distinct types of critical illness myopathy (CIM): (i) myopathy with unspecific morphological alterations [7, 10], (ii) myopathy with selective loss of myosin filaments, i. e. thick-filament myopathy [11–13] and (iii) acute necrotizing myopathy of intensive care [14, 15]. The pathomechanisms of these myopathies, also referred to as acute quadriplegic myopathy or acute myopathy of intensive care, and the relationship to each other still remain un-

clear [16]. Moreover, our knowledge of these conditions is still in its infancy [17]. Basically, these myopathies are believed to be related to sepsis or its systemic inflammatory responses [18]. Cytokines and other factors mediating these responses influence the protein turnover of muscles in various ways resulting in a negative protein balance [19, 20]. Inactivity, denervation and reduced electrical excitability of the muscle membrane may aggravate protein catabolism [18]. In addition to these factors, the occurrence of thick filament myopathy was strongly correlated with the combined use of steroids and neuromuscular blockers [21]. On a subcellular level myosin isoforms have been shown to be subject to calpain-mediated proteolysis in patients with CIM [22]. In a rat animal model, loss of myosin filaments and reduced electrical excitability were produced by glucocorticoid treatment only after denervation of the muscle, by muscle relaxing agents or other pro-inflammatory low-molecular weight mediators in the peripheral blood [23]. An example of such mediators might be peptides and toxins involved in the innate immune response during sepsis (e. g. [24, 25]). So far, studies done in patients with CIM can indicate a more puzzling situation as administration of glucocorticoids and neuromuscular blocking agents has been found not always to result in the development of CIM [26] although these drugs are widely accepted as trigger agents which have been tested on established animal models [27–30]. Moreover, patients given steroids may recover from CIM even when steroids were not discontinued [26]. Also, cytokines (tumour necrosis factor-α and interleukin-6) which it has been suggested are involved in the pathogenesis of CIM [3] were not convincingly increased in the sera of affected patients [31]. Direct electrical stimulation revealed reduced muscular excitability in clinical patients [27]. In an animal model, abnormalities of sodium channel inactivation have been found [28] whereas in animals having undergone high-dose corticosteroid treatment the membrane effects seemed to be more complex [29]. Very recently, inactivation of sodium channels has been correlated with depolarised resting potentials and to be crucially responsible for reduced membrane excitability in the rat animal model of critical illness myopathy for steroid denervated muscle fibres [30]. However, some membrane parameters also indicated an increase in membrane excitability in these animals, especially in denervated muscle fibres without steroid pre-treatment [30].

To further elucidate the pathomechanisms of CIM we studied the effects of blood serum fractions from patients with CIM (i) on the membrane excitability of intact single mammalian muscle fibres as well as (ii) on the excitation-contraction coupling process at the level of the sarcoplasmic reticulum (SR) Ca^{2+} release in chemically skinned muscle bundles. Preliminary results have been presented at the 2002 annual meeting of the German Neurological Society.

Methods

Patients, serum sampling and fractionation, and SDS-Paging

The sera of each 5 volunteer healthy controls (who gave approved consent) and 5 patients with the clinical and neurophysiological diagnosis of CIM according to established criteria [15, 27] were pooled in order to obtain significant amounts of homogeneous material and stored at –20 °C prior to examination. All patients had evidence of sepsis and required mechanical ventilation for more than seven days. During sepsis, patients received 200–300 mg hydrocortisone daily for cortisol replacement. Patients receiving high-dose corticosteroid therapy for the control of bronchial asthma or other indications were excluded from the study. Neuromuscular blocking agents were administered only on an occasional basis as required for tracheal intubation or suction. Blood serum samples were centrifuged using different molecular weight cut-off filters (5, 10, 30 and 100 kDa MWCO, Sartorius, Göttingen, Germany). 1D-Protein electrophoresis (SDS-Gels) was performed using standard procedures [32]. The study was approved by the local Ethical Committee.

Electrophysiology on intact single fibres

To elucidate serum effects on skeletal muscle electrophysiology an established animal model using short murine toe muscle fibres was used [33–35]. Killing of animals (male BALB/c mice of 8–12 weeks of age) and all experimental procedures were carried out according to the guidelines of the local Animal Care Committee. Single short toe muscle fibres (Mm. Interossei) were prepared for Two-Micro-Electrode Voltage Clamp (2-MVC) experiments using enzymatic isolation. To increase the outcome of single fibres the mild collagenase treatment [33, 34] was modified by the addition of protease (0.1 mg/ml, type XXIV, Sigma chemicals) and the incubation was restricted to 25 min at 30 °C. Between experiments single fibres were stored in a refrigerator at 4 °C for up to 8 h.

Resting potentials (E_R), action potentials (AP) and fast sodium inward currents (I_{Na}) were measured under isotonic conditions in normal saline containing (mM): NaCl 144, KCl 4, $CaCl_2$ 2, $MgCl_2$ 1, HEPES 10, glucose, 10, pH 7.4, before (control condition) and after addition of 50 µl aliquots of control serum (CS) or CIM serum fractions to the external solution (final dilution 1:8 –1:10) during the following 60 to 80 min. Some parameters, e. g. E_R, were monitored during the whole incubation time in different fibres and averaged as no systematic run-down could be observed (see time course of Fig. 1 A). E_R values were about –60 mV (range –35 mV to –78 mV) in freshly dissected muscle before enzymatical treatment and somewhat depolarised in single fibres after enzymatic isolation (~ –40 mV, range –25 mV to –64 mV). In some fibres, input resistances R_0 were measured as previously described [35]. The pipette resistance was between 5 MΩ and 7 MΩ when filled with 3 M KCl. In order to be able to elicit action potentials and fast inward I_{Na} currents the single fibres were repolarized to a holding potential of –90 mV using the 2-MVC technique. To optimize space clamp conditions in isotonic solutions short fibres (~ 550 µm) were selected with comparably large diameter (~ 60 µm; see also [33, 35]). The fast activation of Na^+ channels and the relatively high peak Na^+ membrane conductance of mammalian muscle (e. g. ~ 75 mS/cm^2, [36]) require a very fast charging of the membrane capacitance crucially depending on fibre length and diameter [35]. As the I_{Na} currents in the present study (see Figs. 4, 5, see also [35]) are very similar to I_{Na} currents recorded with other techniques, e. g. the vaseline-gap technique [36], the 'loose-patch' clamp technique [37–40] or the Three-Micro-Electrode technique [41] the 2-MVC technique also allows the faithful recording of I_{Na} currents in short fibres.

Skinned fibre experiments

For measurements of Ca^{2+} induced force transients muscle fibre bundles (M. extensor digitorum longus, EDL) from BALB/c mice were dissected in paraffin oil and mounted on a force transducer setting [42]. The preparation was chemically skinned with saponin, the SR loaded and calcium release from the SR induced with caffeine using a standardized procedure [42, 43] while measuring the time course of force transients. Experiments were performed in the absence of serum fractions (controls) and after adding aliquots of 10 µl of control serum (CS) or pooled sera of patients with CIM. The final dilution was 1:50. The force transients during the caffeine activated Ca^{2+} release for different MWCO were recorded and normalized to the maximum force occurring during subsequent maximum Ca^{2+} release in highly activating solution [42, 43]. To record the Ca^{2+} sensitivity of the contractile apparatus the pCa-force (steady state) relations were recorded by Ca^{2+}-activating the fibres at different pCa values between 4.0 and 9.0 in the 50 mM EGTA containing bath solution and relating the steady state force to the pCa. The pCa-curves were fitted with a Hill-equation yielding the flexion point pCa_{50}. All skinned fibre experiments were carried out at room temperature (22–24 °C).

Data analysis

Data acquisition and analysis were performed using pClamp6, AxoTape2.0 (Axon Instr., Foster City, Ca, USA), SigmaPlot5 (Jandel Scientifics, USA) and Origin6 (Microcal, USA) software. Data are given as mean ± SD or ± SEM with number n of observations. Significance was assessed using Student's t-test at the P = 0.05 level.

Results

Membrane effects of CS and CIM serum fractions

Resting membrane potentials ER

Fig. 1 A shows the E_R values after adding control serum (CS, filled circles) or CIM serum (open circles) fractions of 10 kDa (left panel), 30 kDa (middle panel) and 100 kDa (right panel) to the bath solution. During the following incubation time of up to 80 min E_R was measured in several fibres and collected in 10 min interval bins (mean ± SD). The values at time zero (filled square) correspond to E_R measured under control conditions without serum added. From the data it can be seen that after addition of CIM serum E_R seemed to be less negative in most fibres than in controls and during CS incubation especially for 10 kDa and 100 kDa fractions. As E_R did not substantially vary with time, i. e. showed no 'run-down', the data were collected covering the whole incubation time as shown in Fig. 1 B. From $E_R = -38.6 \pm 1.6$ mV in n = 34 control single fibres after enzymatical treatment for fibre isolation, E_R measured -39.6 ± 1.7 mV after the addition of 10 kDa CS serum (n = 16), -35.4 ± 2.1 mV for 30 kDa CS serum (n = 22) and -38.2 ± 0.6 mV after the addition of 100 kDa CS serum (n = 20, ± SEM). Thus, there was no significant change in E_R by CS (P > 0.29). However, E_R became more positive when adding CIM serum (10 kDa: -34.9 ± 1.4 mV, n = 15; 30 kDa: -33.8 ± 1.6 mV, n = 12 and 100 kDa: -31.5 ± 1.2 mV, n = 19, ± SEM) which was not yet significant compared with CS at 10 kDa (P = 0.052) but became highly significant for 100 kDa MWCO (P < 0.001 compared with CS and control). The input resistance R_0 in control fibres was 2.09 ± 0.14 MΩ (n = 15) and was significantly reduced in fibres with 100 kDa CIM serum (0.62 ± 0.19 MΩ, n = 5, P < 0.001).

Serum effects on action potentials (AP)

In order to elicit APs from the same membrane potential all fibres were repolarized to –90 mV by a constant current application. Fig. 2 shows representative recordings of action potentials elicited in the current-clamp mode of the 2-MVC amplifier in two single fibres bathed in normal saline (left panels) and about 3 min after the addition (right panels) of 100 kDa MWCO aliquots of CS (A) or CIM serum (B). The current pulse protocol is shown in the inset. As can be seen from the two examples, the threshold current pulse amplitudes ranged from 200 nA to 300 nA (0.25 ms duration) and were not influenced in the same fibre by the incubation with serum. The examples also show a reduction of AP amplitude to some extent after both the addition of CS or CIM serum. To further quantify serum effects on AP, the following parameters were analysed from the first supra-threshold APs as indicated in the left panel of Fig. 2 A: maximum slope of voltage rise and decay $(^{dV}/_{dt})_{rise}$, $(^{dV}/_{dt})_{decay}$, AP amplitude (ΔA), full width at half maximum (h_w), 10–90 % rise-time (t_r) and 90–10 % decay-time (t_d). The evaluated data are shown for h_w and t_r in Fig. 2, C and D, the others are summarised in Table 1. The data confirm a significant reduction of ΔA for both CS and CIM serum fractions compared with control conditions without serum added as observed in Fig. 2, A and B. However, ΔA seemed to be reduced to similar extents as there was no significant difference between CS and CIM. Similar to ΔA, there was a significant reduction of h_w (Fig. 2 C) and t_r (Fig. 2 D) for both CS and CIM to similar extents for fractions larger than 10 kDa compared to conditions without serum. Furthermore, for 10 kDa MWCO h_w was significantly reduced in CIM compared to CS. In the latter, h_w was similar to controls. $(^{dV}/_{dt})_{rise}$, $(^{dV}/_{dt})_{decay}$ and t_d (Table 1) were also reduced compared with controls after both CS and CIM application for all fractions. However, there was no significant difference between CS and CIM.

Figure 3 shows the effect of CS and CIM serum fractions on the recovery of AP amplitude from experiments eliciting repetitive APs with varying recovery intervals between the pulses. In (A) and (B) repetitive APs are shown in two single fibres before (control condition without serum, left panels) and after the addition of 10 kDa MWCO fractions of CS (A) or CIM serum (B). The pulse protocol is shown in the inset of (A). With de-

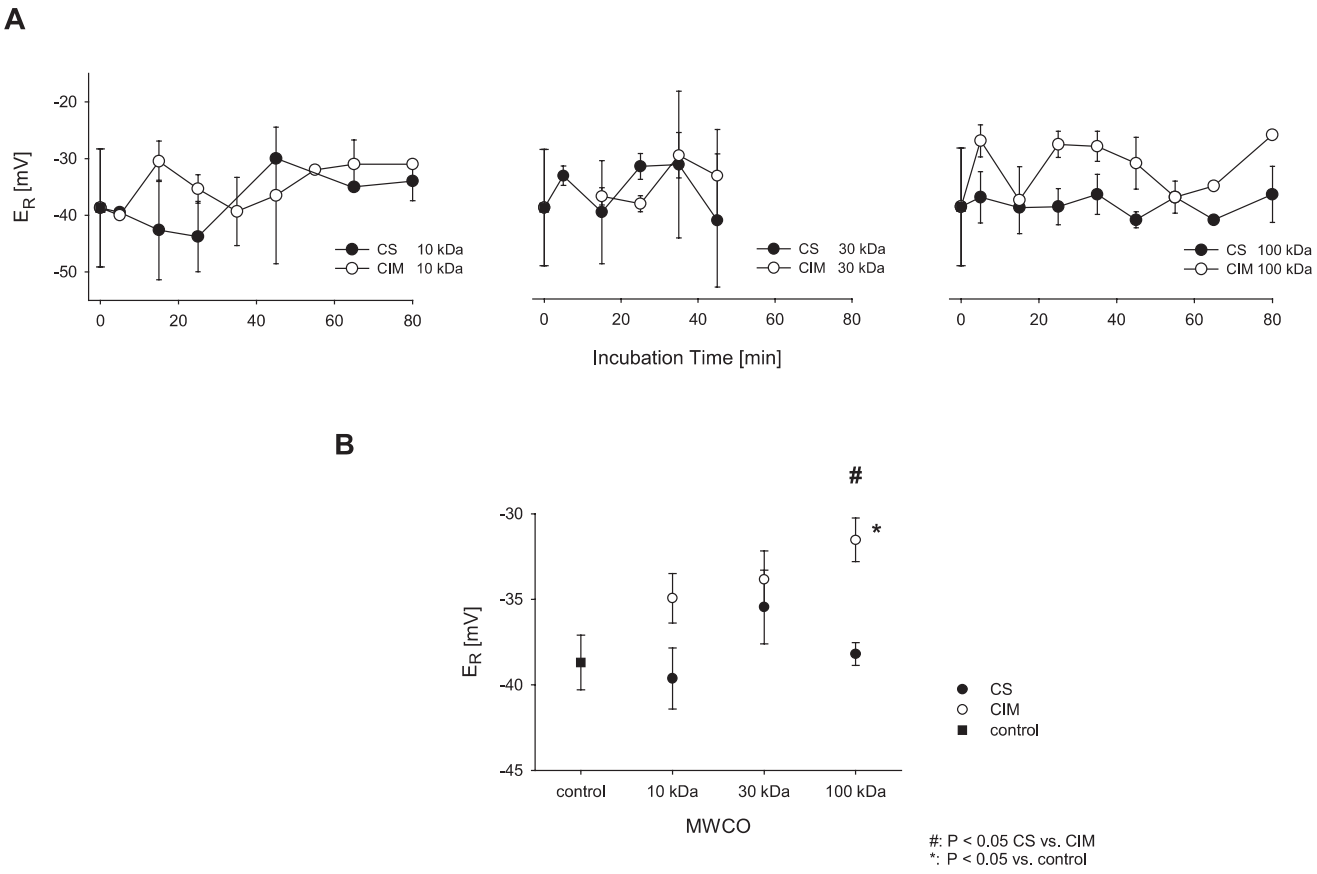

Fig. 1 Effects of CS and CIM serum fractions on the resting membrane potential E_R. **A**, shows the time course of E_R measured in several fibres up to 80 min after the addition of 10 kDa (left panel), 30 kDa (middle panel) and 100 kDa (right panel) fractions of CS (filled circles) or CIM serum (open circles) to the bath solution. E_R values from individual fibres were collected in 10 min intervals and plotted as mean ± SD. E_R at time zero corresponds to values obtained from control fibres in normal saline without serum or prior to serum addition. Note that E_R, in most cases, is more positive after the addition of CIM serum compared to CS. **B**, shows the E_R values collected for each fraction over the whole incubation period. E_R were similar to controls after addition of CS but were significantly reduced after adding CIM serum (#: $P < 0.01$ CS vs. CIM, 100 kDa MWCO)

Table 1 Effects of CS and CIM fractions on AP parameters. Asterisks indicate significance compared with controls without serum

			MWCO [kDa]		
Parameter		Control	10	30	100
$(^{dV}/_{dt})_{rise}$ [mV/ms]	CS	271±14 (n = 14)	237±13 (n = 14)	215±15 (n = 13)	215±19 (n = 7)
	CIM	271±14 (n = 14)	224±22 (n = 13)	224±33 (n = 11)	190±14 (n = 8)
$(^{dV}/_{dt})_{decay}$ [mV/ms]	CS	−150±7	−139±6	−126±9	−118±10*
	CIM	−150±7	−116±11	−120±16	−107±9*
ΔA [mV]	CS	119±2	114±2	109±2*	105±3*
	CIM	119±2	109±3*	108±3*	104±2*
t_d [ms]	CS	0.23±0.004	0.21±0.01	0.19±0.01*	0.19±0.01*
	CIM	0.23±0.004	0.19±0.01*	0.20±0.01	0.20±0.01*

creasing peak-to-peak time (PPT) the amplitude of the second AP (ΔA_2) also decreased as PPT approached the absolute refractory period of the first AP. From the recordings there already seems to be some reduction of ΔA_2 by both CS and CIM which is quantified in Fig. 3 C.

In the left panel the change of the second AP amplitude normalized to the first one ($\Delta A_2/\Delta A_1$) with PPT is shown for six fibres in normal saline without serum (●) and six fibres following addition of 10 kDa CS (■) or CIM serum (□). As indicated in the inset of the right panel in

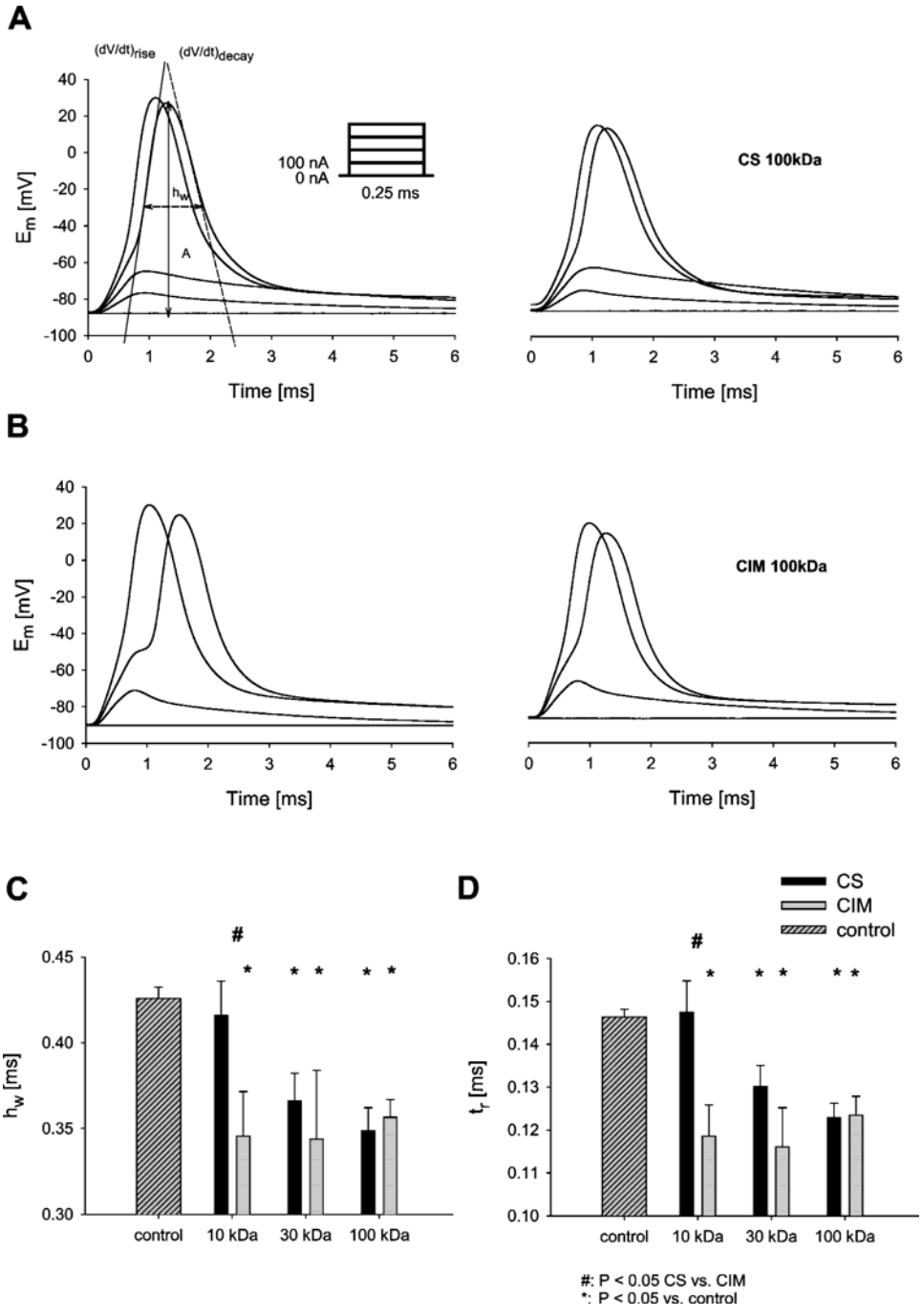

Fig. 2 Effects of CS and CIM serum on action potentials in single fibres. **A**, and **B**, show action potentials elicited by current step pulses of 100 nA increments as indicated in the inset of (**A**) in two single fibres before (left panels) and after the addition of 100 kDa MWCO fractions (right panels) of CS (**A**) or CIM (**B**) serum to the bathing saline. The threshold current amplitude was 200 nA in (**A**) and 300 nA in (**B**) and was unchanged by the addition of serum. AP amplitudes seemed already slightly decreased after the addition of CS or CIM serum. The left panel in (**A**) also visualizes the parameters which were analysed from the AP data (see Table 1). **C**, and **D**, show the evaluated data for the half-width, h_w, and the 10–90 % rise-time, t_r, respectively. For all fractions, both for CS and CIM serum, the values were significantly reduced to the same extent compared with controls in normal saline (*: $P < 0.05$). For 10 kDa, h_w and t_r for CS were significantly larger compared to CIM (#: $P < 0.05$)

(C) for one set of control data (without serum) a time constant τ_{rec} for the recovery and the extrapolated absolute refractory time $(PPT)_{ref}$ could be extracted. For PPT shorter than $(PPT)_{ref}$ no second AP could be elicited. The evaluated data for τ_{rec} are shown in the right panel of (C) for control fibres (hatched bar) in normal saline (no serum added) and after adding the different MWCO fractions of CS (black bars) and CIM serum (grey bars). τ_{rec} was 1.8 ± 0.3 ms in control fibres in normal saline ($n = 6$, ± SEM). There was no significant difference in τ_{rec} after the addition of CS or CIM fractions ($P > 0.17$). Also the estimated $(PPT)_{ref}$ did not seem to be systematically changed by addition of either serum ($P > 0.3$, data not shown).

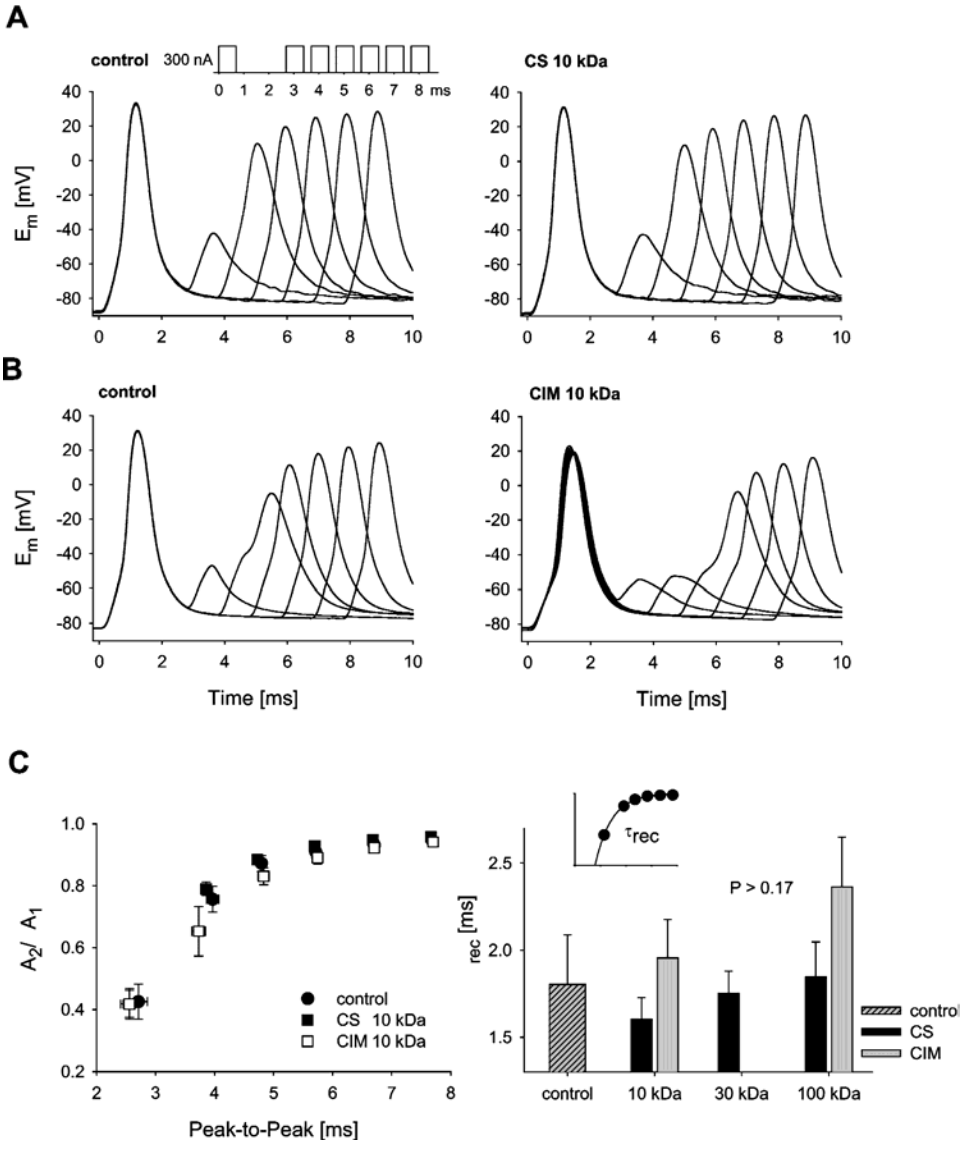

Fig. 3 Effects of CS and CIM serum on the recovery of repetitive action potentials. **A** and **B**, show representative recordings of action potentials elicited with a double pulse protocol as shown in the inset of (**A**) with varying inter-pulse duration in two single fibres prior to (left panels, control) and after the addition of 10 kDa fractions of CS (**A**) or CIM serum (**B**). The amplitude of the second AP (ΔA_2) was related to that of the first AP (ΔA_1) and plotted against the peak-to-peak time (PPT) as shown in the left panel of (**C**) for six control fibres recorded under the same condition as in (**A**) and (**B**). The data could be well fitted with a single exponential (as shown in the inset of the right panel) yielding the time constant τ_{rec} of the amplitude recovery and the estimated absolute refractory time for successive APs. τ_{rec} is evaluated for 10 kDa, 30 kDa and 100 kDa serum fractions in the right panel of (**C**) on several fibres each

Serum effects on sodium currents (I_{Na})

The observed changes in the AP parameters could be due to changes in the sodium channel activation or inactivation characteristics. We therefore examined the effects of CS and CIM serum fractions on the peak amplitudes of I_{Na} and the voltage dependent inactivation h_∞ of I_{Na}. Fig. 4 A shows representative recordings of I_{Na} from a single fibre under control conditions in normal saline (left panel) and after the addition of a 10 kDa CS fraction. I_{Na} was elicited from a holding potential of –90 mV in isotonic normal saline containing 5 mM TEA^+ to suppress K^+ outward currents. I_{Na} was activated by 10 mV increments for 7.5 ms as shown in the inset. Fig. 4, B - D, shows the resulting I_{Na}-V plots for control fibres in normal saline (●, B–D) and following the addition of 10 kDa (B, CS: ■, CIM: □), 30 kDa (C, CS: ▲, CIM: △) and 100 kDa (D, CS: ▼, CIM: ▽) serum fractions. Threshold potentials were similar under all conditions and there was no significant change in maximum peak I_{Na} for CS (10 kDa: –885 ± 58 nA, n = 5, 30 kDa: –717 ± 86 nA, n = 8, 100 kDa: –873 ± 53 nA, n = 12) compared with controls (–854 ± 55 nA, n = 13, ± SEM, $P > 0.17$). However, maximum peak I_{Na} was significantly larger after the incubation with 10 kDa CIM serum (–1039 ± 53 nA, n = 7, $P < 0.05$) than in controls and showed a tendency to decrease for larger fractions (30 kDa: –907 ± 100 nA, n = 4, 100 kDa: –829 ± 64 nA, n = 16) becoming similar to controls. There was a tendency for maximum peak I_{Na} to be larger for 10 kDa and 30 kDa MWCO CIM fractions compared with CS fractions, although this was not significant ($P > 0.08$).

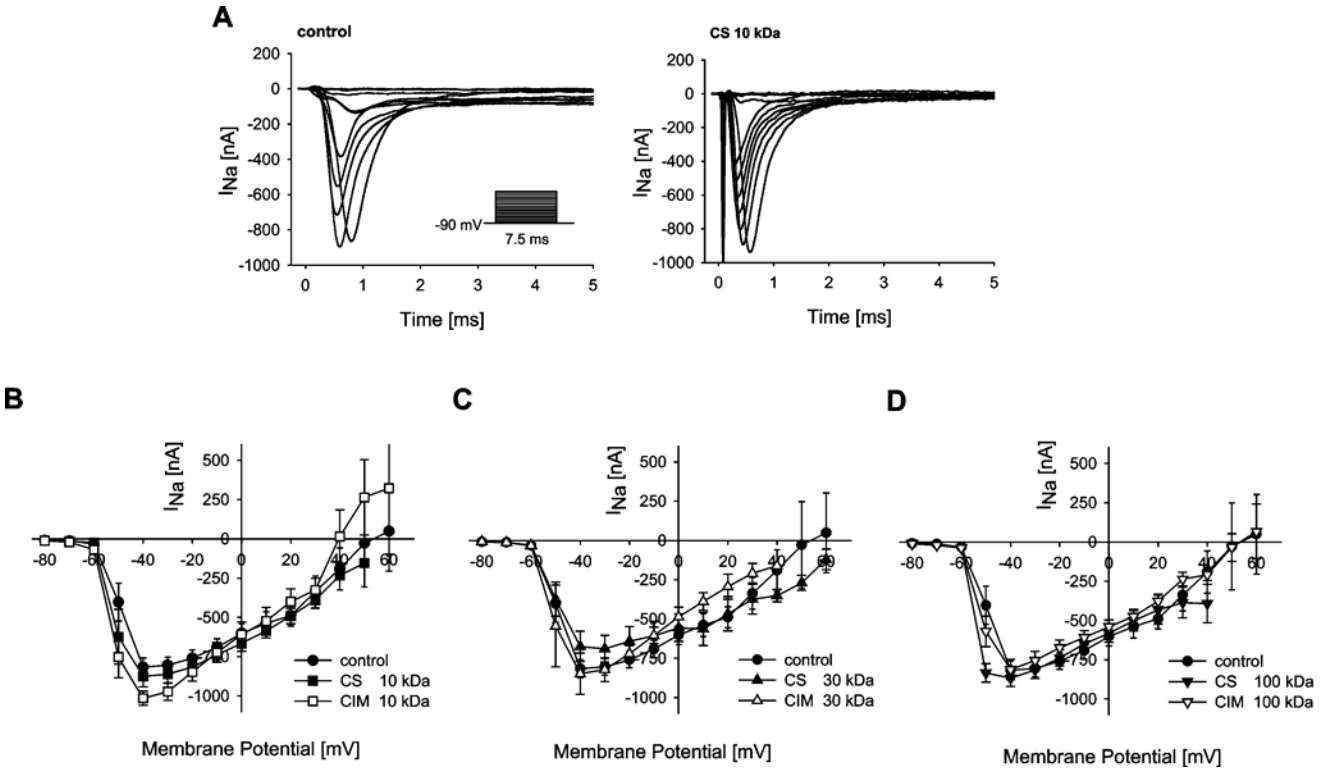

Fig. 4 Effects of CS and CIM serum on the activation of I_{Na}. **A** shows a complete set of I_{Na} traces recorded in a representative single fibre before (control, left panel) and after the addition of 10 kDa CS. Inward I_{Na} were elicited from a holding potential of –90 mV with a pulse protocol as shown in the inset. Outward K^+ currents were blocked by TEA^+ in the external solution. **B–D** show the I_{Na}-V-relations recorded from several control fibres (filled circles in *B–D*) and after the addition of 10 kDa (**B**), 30 kDa (**C**) and 100 kDa (**D**) fractions of CS (filled symbols) or CIM serum (open symbols). Maximum I_{Na} amplitudes were increased after addition of CIM serum. See text for details

The voltage-dependent inactivation of I_{Na}, h_∞, is evaluated in Fig. 5 from double-pulse recordings as shown in (A) for a representative single fibre after addition of 10 kDa CS (pulse protocol in the inset). h_∞ (right panel) was derived from the current traces (left panel) by relating the relative I_{Na} peak amplitude during the second pulse to the pre-pulse potential. The solid line represents the Boltzmann-fit to the data yielding the half-inactivation potential $h_{0.5}$ and the steepness k [35]. The h_∞ plots for n = 4 fibres after incubation with 10 kDa CS (filled symbols) and 10 kDa CIM (open symbols, n = 5) are superposed in (B) with the mean Boltzmann fits (CS: solid line, CIM: dashed line). For this MWCO fraction CIM serum shifted h_∞ about 6 mV ($h_{0.5} = -68.9 \pm 1.6$ mV) to more positive potentials than CS ($h_{0.5} = -74.6 \pm 3.5$ mV, $P < 0.05$). For 100 kDa this significant difference was also present (CS: $h_{0.5} = -77.1 \pm 7.1$ mV, CIM: $h_{0.5} = -69.3 \pm 7.4$ mV, $P < 0.05$). Interestingly, $h_{0.5}$ values were similar for 10 kDa fractions (CS: $h_{0.5} = -70.7 \pm 6.4$ mV, CIM: $h_{0.5} = -73.5 \pm 5.0$ mV, $P > 0.45$, Fig. 5 C). However, for all fractions of both CS and CIM $h_{0.5}$ was not significantly different from control conditions without serum added ($h_{0.5} = -69.3 \pm 7.4$ mV, $P > 0.11$). As can be seen from Fig. 5 C, $h_{0.5}$ showed a tendency to increase with MWCO for CS whereas it decreased for CIM.

Ca^{2+} release from the SR and Ca^{2+} sensitivity of the contractile apparatus

To elucidate, if CIM serum impairs the excitation-contraction coupling process at the level of the SR Ca^{2+} release or the Ca^{2+} sensitivity of the contractile apparatus, a 'skinned fibre' preparation was used which allows incubation of the contractile apparatus with serum fractions after chemical permeabilisation of the plasma membrane [42, 43].

Fig. 6 shows representative recordings of caffeine activated Ca^{2+} induced force transients in skinned fibres (A) under control conditions before (solid line) and after the addition of CS (dashed line) or CIM (dotted line) serum of 10 kDa MWCO. The force transient in (A) was normalized to the maximum force recorded during subsequent maximum calcium release in highly activating solution (HA plus caffeine, [43]). Thus, the force transients between different fibre bundles could be compared. To investigate the calcium sensitivity of the contractile apparatus the force development at different pCa values in the bathing solution was recorded. A pCa-force recording after CIM serum incubation (100 kDa) is shown in (B). The pCa-force relations of a fibre bundle under control (no serum added) vs. CS condition and

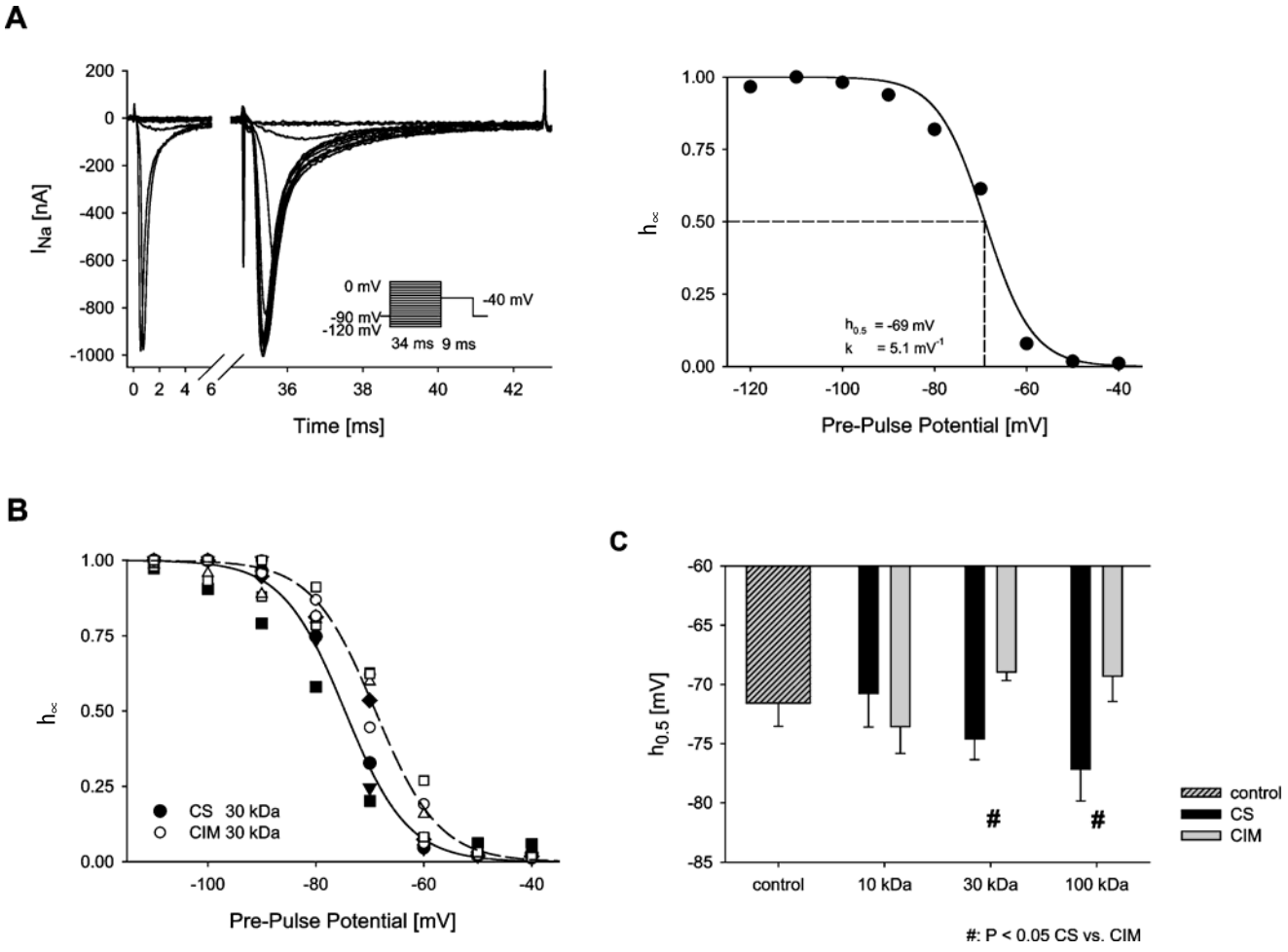

Fig. 5 Effects of CS and CIM serum on the voltage dependent inactivation h_∞ of I_{Na}. **A** left panel, shows I_{Na} recordings in a representative fibre after addition of 10 kDa CS with a double-pulse protocol as shown in the inset. Depending on the pre-pulse potential the availability of sodium channels for the second pulse decreased. The corresponding h_∞ plot for this fibre is shown in the right panel. The solid line represents the corresponding Boltzmann-fit. **B** shows h_∞ plots from n = 4 fibres after the addition of 30 kDa CS serum (closed symbols) and n = 5 fibres for 30 kDa CIM serum fractions (open symbols). The lines represent the mean Boltzmann-fits (CS: solid line, CIM: dashed line). **C** shows the mean half-inactivation potentials $h_{0.5}$ for control fibres and the different serum fractions of CS and CIM. For CIM serum larger than 10 kDa $h_{0.5}$ was significantly reduced compared to CS (#: P < 0.05)

control vs. CIM (both 100 kDa MWCO) are shown in the left and right panel of Fig. 6, C and D, respectively. The Hill-fit to the data was virtually unchanged by addition of either CS (solid line) or CIM serum (dashed line) of 100 kDa MWCO. Figure 6 E shows the evaluated mean peak force values of Ca^{2+} induced force transients from n = 14 fibres in normal saline (control condition) and after addition of CS or CIM serum fractions (5 kDa to 100 kDa MWCO, ± SEM, n between two and seven). For lower MWCO up to 10 kDa the peak force was significantly increased by CS (*: P < 0.05) compared to the control condition before adding the serum fraction. Interestingly, for 5 kDa MWCO peak force was significantly reduced by CIM serum compared with CS. However, it increased to values similar to CS at 10 kDa. For higher MWCOs, e. g. 30 kDa and 100 kDa, the peak force markedly decreased to values similar to control conditions for both CS and CIM. Figure 6 F shows the corresponding evaluation of the pCa_{50} values from the pCa-force relations. CS and CIM serum had similar effects on the pCa-force relation as pCa_{50} was not significantly different between the two sera for all fractions. Interestingly, serum 'per se' seemed to decrease the pCa_{50} values with increasing MWCO. This cannot simply be explained by an increase of the Ca^{2+} buffering capacity in the recording solution after adding serum fractions of larger MWCO as the calculated final albumin concentration under our experimental conditions (< 0.05 mM) will not alter the maximum Ca^{2+} buffer capacity set by 50 mM EGTA (see. Methods).

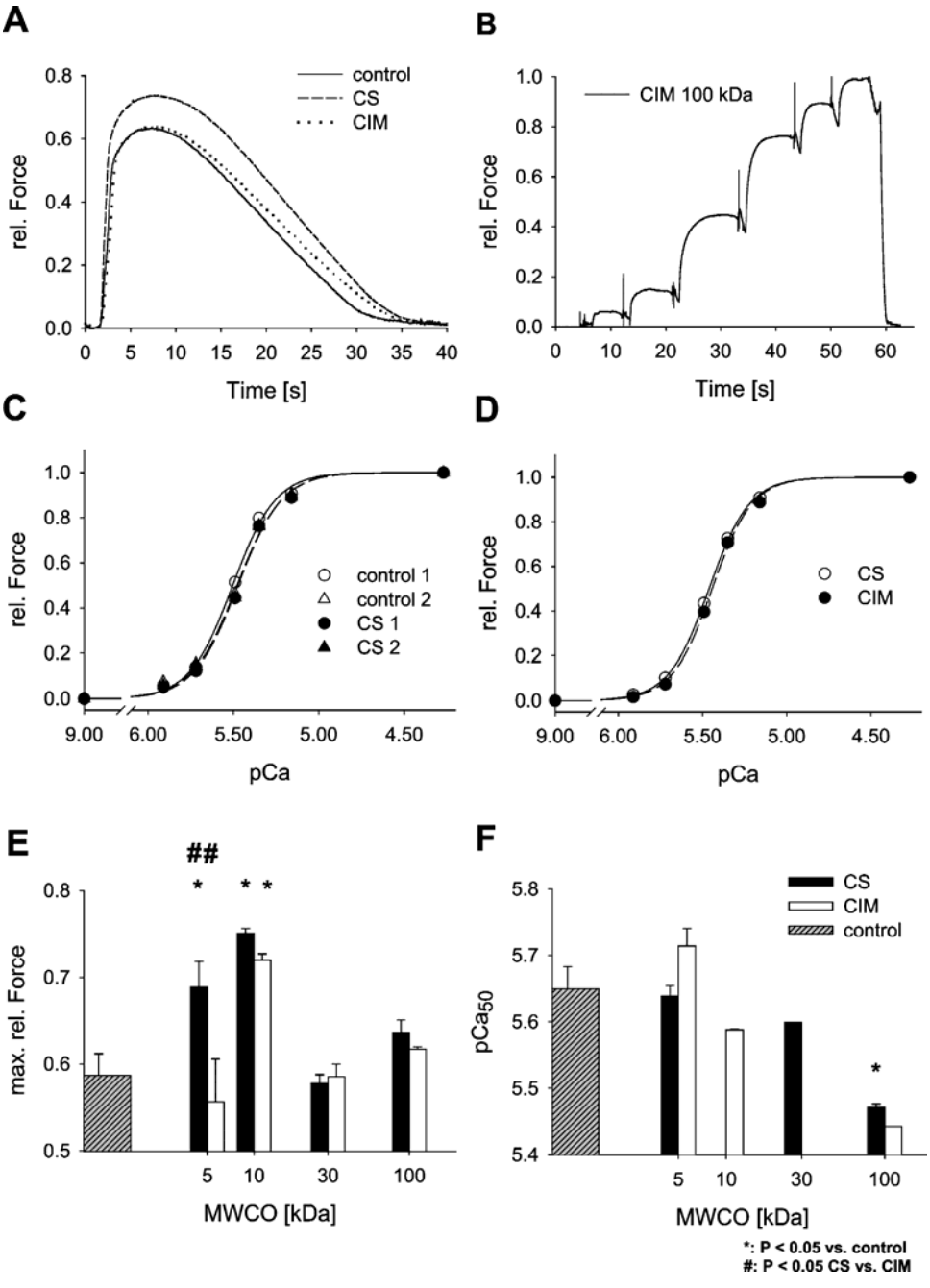

Fig. 6 Caffeine induced Ca^{2+} release from the SR and Ca^{2+} sensitivity of the contractile apparatus in skinned fibre bundles incubated with CS and CIM serum fractions. **A** shows representative recordings of caffeine-activated Ca^{2+} induced force transients under control conditions (solid line) and after adding 10 kDa fractions of CS (dashed line) or CIM serum (dotted line) to the bath solution. **B** shows recordings of force when exposing the preparation to solutions with decreasing pCa for a fibre bundle after addition of 100 kDa MWCO CIM serum to the solutions. **C** and **D** show the evaluated pCa-force relations from the steady-state force of recordings as in (**B**) and following incubation with CS (**C**) or CIM serum (**D**). The lines represent least square Hill-fits to the data (**C:** solid line for controls, dashed line for CS, **D:** solid line for CS, dashed line for CIM). For both CS and CIM there was no change in the pCa-force relation. **E** shows the mean peak amplitudes of caffeine-activated Ca^{2+} induced force transients for n = 14 control fibres and after incubation of 5 kDa (n = 11, n = 11), 10 kDa (n = 3, n = 2), 30 kDa (n = 7, n = 4) or 100 kDa (n = 2, n = 2) MWCO CS or CIM fractions, respectively. **F** shows the mean pCa_{50} values (n = 10 control fibres, 5 kDa: n = 2, n = 9; 10 kDa: n = 0, n = 2; 30 kDa: n = 1, n = 0; 100 kDa: n = 3, n = 1). *: P < 0.05 vs. control, #: P < 0.05 CS vs. CIM, ± SEM

SDS-Gels from control and CIM serum fractions

To clarify whether the changes seen in membrane and contractile function induced by CIM serum fractions may be explained by a protein factor, 1D-SDS gel electrophoresis was performed. SDS silver-chloride stained gels of CS and CIM sera showed no major differences in the protein bands for all cut-offs tested (Fig. 7). The low-molecular weight proteins (< 35 kDa) in the 100 kDa MWCO bands are fragments of the > 50 kDa portion as they are not present in the bands from smaller cut-offs (10 kDa, 30 kDa).

Discussion

In critically ill patients, 'de novo' arising myopathies are one of the main causes for secondary neuromuscular complications and can be potentially hazardous by prolonging the time on the respirator and for rehabilitation.

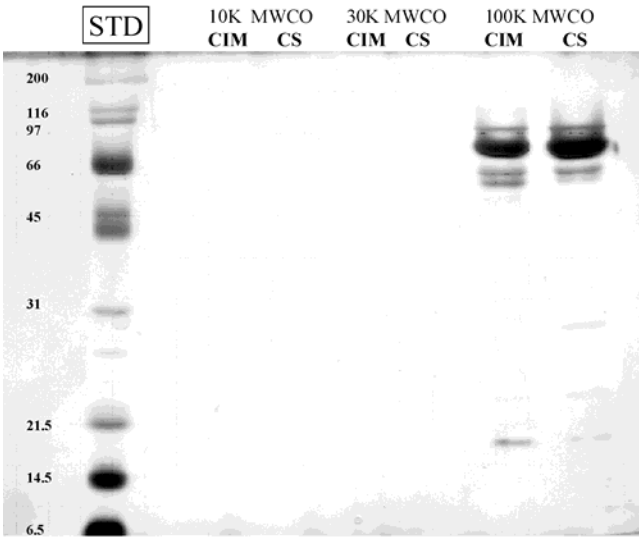

Fig. 7 SDS Gels of 10, 30 and 100 kDa MWCO serum fractions from CIM patients (CIM) and control persons (CS). For all cut-offs no major difference between the control and patient group can be detected

Specific studies on the mechanisms by which muscle function is compromised have not been done. The present study was designed to systematically investigate the effects of different molecular weight fractions of CIM serum on membrane excitability, ion conductances and calcium turnover in the SR.

Serum effects on the muscle membrane

Addition of CIM serum caused a marked depolarisation of E_R for all fractions tested whereas CS serum did not seem to affect E_R. In primary cultures of human atrial myocytes the presence of serum in the culture medium has also been shown to result in lower resting potentials compared with freshly dissociated cells [44]. One explanation for the reduction in E_R after incubation with CIM serum fractions, especially for 100 kDa MWCO, could be that a resting conductance component with a reversal potential less negative than the resting potential is induced by this serum fraction. Alternatively, this is also compliant with a loss of function of hyperpolarising ion channels (i. e. Cl^- and K^+). For the input resistances R_0 a significant reduction was found when adding CIM serum to normal saline. This is a particularly interesting finding, as in an animal model for acute quadriplegic myopathy, R_0 in EDL fibres of mice having undergone prolonged high dose corticosteroid administrations was also found to be decreased compared to controls, although to a lesser extent [29]. However, in the same study the authors found only slightly reduced (more positive) E_R values than in controls. In contrast, in neonatal rat cardiac myocytes, free long chain fatty acids shifted E_R about 5 mV to more negative values compared with controls within 2–5 min [45] and no effect on E_R was found in mouse muscle after intraperitoneal injections of patient myasthenia gravis serum [46].

In our preparation, both CS and CIM serum had marked MWCO dependent effects on the properties of APs. In summary, AP amplitudes were significantly reduced and AP rise and decay kinetics were accelerated compared to controls in normal saline by application of both serum types to similar extents (table 1). However, the narrowing of APs as judged from the half-width and the accelerated rise-time were even more pronounced in CIM serum compared with CS serum especially for low molecular fractions (10 kDa, Fig. 2). On the other hand, the recovery from these accelerated action potentials was prolonged for CIM serum compared with control and CS (Fig. 3). Influences of sera or their fractions on AP kinetics and amplitudes are diverse in different preparations and also depend on the incubation period. For example, acute exposure to fatty acids has been described to reduce membrane excitability in cardiac myocytes by increasing the threshold for action potentials and the intervals between spontaneous action potentials [45]. Additionally, phase 4 of cardiac depolarisation was slowed down. These effects could be reversed by binding of fatty acids to serum albumin. However, in rabbit heart cells [47] or murine muscle [48] short-term incubation with serum fractions from anti-Ro/SSA positive patients or myasthenia gravis patients, respectively, did not affect action potential parameters. In cultured neuronal cells, long-term exposure to serum albumin and fatty acids in the culture media produced a 40 % decrease in the maximum rise of the action potential and a small change in AP amplitude [49], whereas Rich et al. [29] found reduced amplitudes of compound action potentials in their animal model (rat tibialis anterior muscle) following high dose steroid therapy.

Our findings on AP kinetics suggest that certain serum fractions from patients with CIM influence voltage gated channels, i. e. the sodium channels. CIM serum, especially low molecular fractions, caused an increase in I_{Na} amplitude in our preparation both compared with controls in normal saline and after administration of CS serum with otherwise no major changes in the I-V-relationship (Fig. 4). This could be explained by a shift of the voltage dependent inactivation curve, h_∞, to more positive potentials for CIM compared to CS serum. Interestingly, CS itself already produced a shift of h_∞ in the opposite direction compared to controls in normal saline. Similar results have been obtained in human myoball preparations [50]. The authors also found an increase in Na^+ current amplitudes induced by incubation with serum from patients with Guillain-Barré syndrome. They also found a shift of h_{oc} to more positive potentials. On the other hand, incubation with cere-

brospinal fluids had the opposite effect [50]. A block of Na^+ channels was also found in nerve cells incubated with anti-GM1 antibodies and was described to be due to changes in membrane surface charge [51]. Interestingly, in a rat animal model of critical illness myopathy, Rich & Pinter recently found the inexcitability of muscle to be crucially related to a hyperpolarizing shift of fast inactivation of sodium channels at depolarised membrane potentials under their experimental conditions where rats had been treated by high-dose steroid applications and denervation [30]. However, they also found some changes to Na^+ channel activation and slow inactivation which seemed to increase rather than decrease membrane excitability [30]. These effects might be specific for steroid treated and denervated muscle fibres whereas our approach might more specifically reflect the sepsis related contribution to the pathomechanism of CIM.

Serum effects on the muscle excitation-contraction coupling process

The excitation-contraction coupling process of EDL muscle at the level of the SR Ca^{2+} release was also modified by low molecular weight fractions of both CIM and CS. The most pronounced effect could be seen from fractions up to 10 kDa where the peak of the caffeine-activated Ca^{2+} induced force transients was significantly increased by CS compared to control conditions without serum although the calcium sensitivity of the contractile apparatus only showed minor changes. However, CIM serum at 5 kDa produced markedly decreased peak values of the Ca^{2+} induced force transients compared to those obtained in the presence of CS. This might be explained by an inhibition of calcium release channels induced by low molecular fractions in CIM serum. Interestingly, these effects were completely reversed at higher fractions. However, this is unlikely to be due to a substantial increase of Ca^{2+} binding (Ca^{2+} buffering) by albumin or other high molecular proteins [45] as under our experimental conditions using a final dilution of 1:50 for 'skinned fibre' recordings and assuming an albumin concentration of ~ 1 mM (6–8 g/dl) the maximum Ca^{2+} binding capacity (50 mM EGTA in the recording solution) would hardly change. It is further interesting to note, that a calculation of total free calcium in serum fractions containing e. g. albumin seems difficult and may be even inappropriate as calcium binding to albumin is a complex process characterised by multiple binding sites with variable affinities and binding capacities [52]. Alternatively, a 'myotoxic' CIM inducing factor in the low molecular serum fractions might be inactivated by direct binding to albumin [45]

As low molecular fractions, especially lipophilic agents, may well pass the plasma membrane such substances may be in part responsible for the muscle weakness in patients with CIM. Interestingly, for myasthenia gravis serum no effects on the muscle mechanical properties have been found [46].

In conclusion, our data demonstrate differential effects of CIM serum on resting and action potentials, sodium channels as well as the excitation-contraction coupling process at the level of the SR-Ca^{2+} release which seem to be more pronounced for low molecular fractions. A humoral factor of low molecular weight has been proposed to be responsible for the development of critical illness polyneuropathy [53, 54]. Also, a low molecular endogenous protein acting as a sodium channel blocker, ultimately turning out to be a pentapeptide, has been identified in the cerebrospinal fluid of patients with inflammatory autoimmune disorders of the nervous system [55]. Other authors have also postulated a factor inducing myopathy in the serum of patients suffering from CIM [31]. The factor causing the effects observed in our experiments might be a small polypeptide or a lipophilic substance, e. g. a fatty acid, as protein SDS pages did not show differences between CIM and control serum. Fatty acids are known to alter membrane properties [45, 46, 48, 49] and can be antagonised by serum albumin [45]. Furthermore, possible candidates might also be considered from pro-inflammatory mediators such as toxins released by immune response activated cells of the innate host defense [24, 25]. Such factors might also be involved in changes to sodium channel gating by phosphorylation or glycosylation [30, 56, 57].

Taken together our data provide a first evidence for a humorally mediated factor which might be involved in the pathomechanism of CIM. They also indicate that even control serum fractions 'per se' alter the response to important physiological membrane and contractility parameters compared with physiological saline. Therefore, the present study might also reflect a more physiological condition of the preparations from which the data are obtained.

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