Slow and Fast Fatigable Frog Muscle Fibres: Electrophysiological and Histochemical Characteristics

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Abstract. Continuous activity of isolated frog gastrocnemius muscle fibres provoked by repetitive stimulation of 5 Hz was used as an experimental model for fatigue development in different fibre types. Parameter changes of the elicited intracellular action potentials and mechanical twitches during the period of uninterrupted activity were used as criteria for fatigue evaluation.

Slow fatigable muscle fibre (SMF) and fast fatigable muscle fibre (FMF) types were distinguished depending on the duration of their uninterrupted activity, which was significantly longer in SMFs than in FMFs. The normalized changes of action potential amplitude and duration were significantly smaller in FMFs than in SMFs. The average twitch force and velocity of contraction and relaxation were significantly higher in FMFs than in SMFs.

Myosin ATPase (mATPase) and succinate dehydrogenase activity were studied by histochemical assessment in order to validate the fibre type classification based on their electrophysiological characteristics. Based on the relative mATPase reactivity, the fibres of the studied muscle were classified as one of five different types (1-2, 2, 2-3, 3 and tonic). Smaller sized fibres (tonic and type 3) expressed higher succinate dehydrogenase activity than larger sized fibres (type 1-2, 2), which is related to the fatigue resistance.

The differences between fatigue development in SMFs and FMFs during continuous activity were associated with fibre-type specific mATPase and succinate dehydrogenase activity.

Key words: Peripheral muscle fatigue — Intracellular action potential — Twitch — Muscle fibre types — Myosin ATPase — Succinate dehydrogenase

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Introduction

Fatigue is a process of a changed muscle functional state. Two main causes are thought to underlie fatigue in isolated muscle preparations: 1. failure of the activation system to generate and propagate action potentials, and 2. reduced capacity of the myofibrils to produce force at any given state of activation (Edman 1995). During repetitive stimulation diminished membrane excitability predetermines i) a failure of excitation-contraction coupling (Eberstein and Sandow 1963; Grabowski et al. 1972); ii) a decrease in amplitude and an increase in duration of the action potential (Jones 1981; Metzger and Fitts 1986; Mileva et al. 1998); and iii) a specific pattern of muscle fibre activity consisting of alternating periods of failure and reappearance of action potential (Mileva and Radicheva 1996; Radicheva et al. 1998). Repetitive stimulation is associated with a disturbance in sarcolemma and T-system membrane excitability caused by modification of functional properties of ionic channels as well as changes in ionic gradients of Na^+, K^+, Cl^- and Ca^{2+}. During high-frequency stimulation there is a rapid loss of force accompanied by a slowing of the action potential and an increase of excitation threshold (Fuglevand 1995). Alterations in single muscle fibre intracellular action potentials (ICAPs) reflect the changes in the ionic currents and ionic conductivity in the excitable membrane (Gydikov 1992).

Vertebrate skeletal muscles are composed of a variety of different fibre types that exhibit large differences in their mechanical and energetic properties. One of the widely accepted muscle fibre classification systems is based on myosin adenosine triphosphatase (mATPase) profile of the fibres (Brooke and Kaiser 1970a,b). Many contractile characteristics of the muscle are defined by the myosin isoforms expressed within the fibres. The combinations of myosin heavy chains (MHCs) and myosin light chains (MLCs) provide specific force- and velocity-dependent properties, mechanical power production capabilities, end energetic cost of force production (Curtin and Davies 1973; Sweeney et al. 1986; Bottinelli et al. 1994; Lutz et al. 2002). Different fibre types in amphibian muscles contain different myosin isoforms (Rowlerson and Spurway 1988; Lutz et al. 1998a,b). The fibres of Ranid skeletal muscles have been classified into three twitch fibre types: 1, 2 and 3, and one tonic type based on the different pH lability of the mATPase systems. The classification was verified by immunoreactivity of the fibres (Lutz et al. 1998a,b). Type 1 and tonic fibres exhibit positive reaction under acid preincubation conditions, while types 2 and 3 fibres are inhibited. Under alkaline preincubation conditions, only type 2 fibres are stained. The mitochondrial enzyme succinate dehydrogenase (SDH) is widely used as a metabolic marker for the oxidative processes, which is correlated to the fatigue resistance (Lńnnergren and Smith 1966; Burke et al. 1973; Kugelberg and Lindergren 1979; Westerblad and Lńnnergren 1986; Spurway and Rowlerson 1989). Due to multiple innervation of the frog muscle fibres, composing most of the motor units (MUs), an analogy with the well-known mammalian MUs' characteristics is inappropriate (Hunt and Kuffler 1954). Therefore, we applied imposed activity to isolated muscle fibres in order to analyse fibre type dependency

of fatigue development. In our previous studies performed on isolated frog muscle fibres, the rate of action potential propagation velocity (PV) changes for the duration of the period of uninterrupted activity (named endurance time, ET) have been used as a criterion for distinguishing the fibre types (Radicheva et al. 1998). The fibres were classified conditionally into two groups – slow fatigable muscle fibres (SMFs) and fast fatigable muscle fibres (FMFs) depending on the calculated normalised PV change for the corresponding ET, which was significantly different for both fibre types.

The present investigation could be considered as an experimental model for elucidation of fibre type specific alterations in electrical and mechanical activity caused by fatigue progressing. The aim of this study was to correlate parameter changes of muscle fibre ICAPs and twitches during fatiguing activity to the fibre type specific properties established by histochemical assays of mATPase and SDH activities. Acid and alkaline preincubation conditions were used to determine fibre type composition of the investigated muscle.

Materials and Methods

Experimental protocol

The experimental set-up is illustrated in Fig. 1. The experiments were performed on adult *Rana ridibunda* following the institutional ethical guidelines. The frogs were killed by double pithing and both gastrocnemius muscles were removed immediately. Bundles of muscle fibres dissected from the muscles were immersed in standard Ringer's solution containing (in mmol/l): 115 NaCl, 2 KCl, 1.8 $CaCl_2$, 0.68 NaH_2PO_4, and 1.3 Na_2HPO_4 (pH 7.3), and mounted horizontally in a temperature-controlled (20–21 ℃) double-wall chamber as it was described earlier (Radicheva et al. 1999).

Repetitive suprathreshold stimulation (double the corresponding threshold for each fibre) with rectangular pulses of 0.5–1 ms duration and intersimulus interval of 200 ms (5 Hz) was applied *via* a functional generator (Anapulse stimulator, model 305-1, WP Instruments Inc.) with stimulus isolation unit (model 302-T, WP Instruments Inc.). The ICAPs were obtained by means of pyrex glass microelectrodes with a tip diameter of 1 μm and a resistance of 15–20 MΩ, filled with 3 mol/l solution of KCl. The microelectrode was connected with a recording system (model M-707 Micro-probe system, WP Instruments Inc.) by $Ag/AgCl_2$ electrode. In order to suppress the mechanical activity of the fibres 270 mmol/l sucrose was added to the Ringer's solution.

The mechanical activity (contraction curves, twitches) was examined in another experimental series using the same stimulation scheme on single muscle fibres stretched to a length at which the suprathreshold single stimulus pulse evoked a maximal response. Twitches were recorded *via* a force transducer and a 5 KHz carrier frequency amplifier for a KWS 3082A strain gauge (Hottinger Baldwin Messtechnik) in absence of sucrose in the surrounding medium.

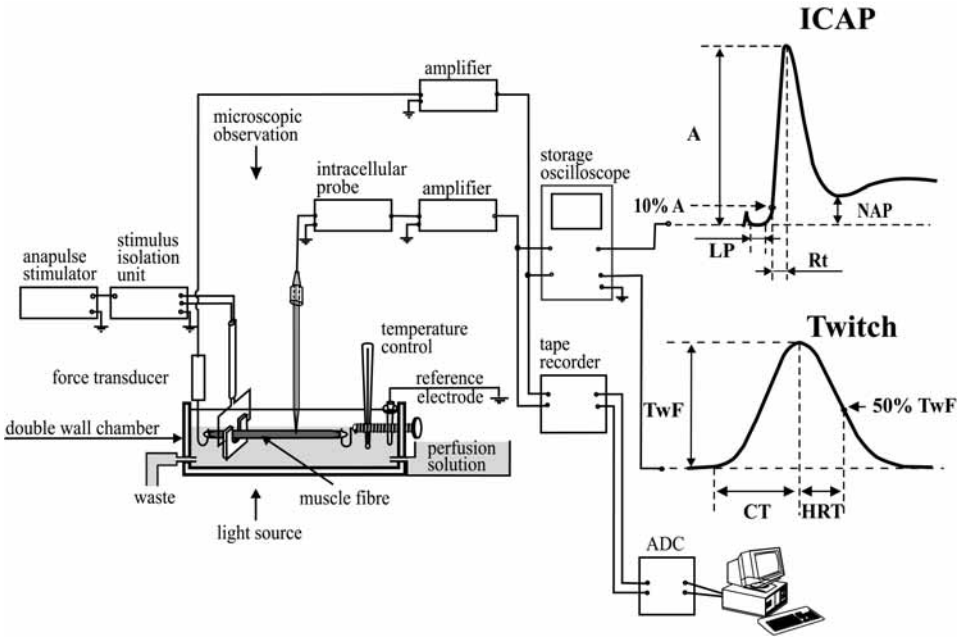

Figure 1. Block-scheme of the experimental set-up and the recorded signals. Intracellular action potential (ICAP) parameters: amplitude of the potential (A); rising time (Rt) – time interval between rising phase of the potential at level 10 % of A and the peak of depolarization phase; amplitude of the negative afterpotential (NAP); latency period (LP) – time interval between the end of the stimulus artefact and the initial deflection of the ICAP waveform. Twitch parameters: force (TwF); contraction time (CT) – time interval between the beginning of twitch rising phase and the peak of the contraction; half relaxation time (HRT) – time interval between the peak of the contraction and the twitch falling phase at level 50 % of TwF. ADC, analogue-to-digital converter.

Data analysis

The obtained ICAPs and twitches were transferred on-line to a computer *via* an analogue-to-digital converter (CED Power 1401, Cambridge, UK) with a sampling frequency of 12,500 Hz *per* channel and an accuracy of sampling of 12 bits. Off-line data analysis was performed using custom-written scripts developed in CED software Spike2 (Cambridge, UK).

The ICAP and twitch parameters (Fig. 1) used to characterize the effect of repetitive activity were measured at the beginning (first action potential and twitch) and at the end (last action potential and twitch) of the ET, which was different for each muscle fibre. The ICAP parameters measured were as follows: amplitude of the potential (A); rising time (Rt) – the time interval between rising phase of the potential at level 10 % of A and the peak of depolarization phase; amplitude of the negative afterpotential (NAP), measured as the amplitude be-

tween ICAP repolarization minimum to the baseline; latency period (LP) – the time interval between the end of the stimulus artifact and the initial deflection of the ICAP waveform. In order to avoid errors in determination of the depolarization onset due to base line fluctuations, the measurement of Rt was set to start at the time point of 10% of ICAP amplitude as previously suggested (Wallinga-De Jonge et al. 1985). Due to the disproportionate change of the A and the NAP during the trial, the NAP/A ratio was used as a more informative parameter. The twitch parameters considered were: twitch force (TwF); contraction time (CT) – the time interval between the beginning of twitch rising phase and the peak of the contraction; half relaxation time (HRT) – the time interval between the peak of the contraction and the twitch falling phase at level 50% of TwF; average rates of the TwF rise and decline (TwF/CT and TwF/HRT, respectively). The change of each parameter (Δ [%]) was calculated as the difference between the last and the first potential (twitch, respectively) values during ET, normalised against the first potential (twitch) parameter value. Data are plotted as means $\pm$ standard errors of the mean (SEM).

The SMFs and FMFs, classified in our earlier studies into two groups by Cluster analysis of their rates of PV change, demonstrated completely different ET (Radicheva et al. 1998). Based on this finding, the duration of muscle fibre uninterrupted activity (ET) was used in the present investigation as a criterion for distinguishing the fibre types.

A one-factor repeated-measures analysis of variance (ANOVA) was used to test for difference among the parameter values between the two fibre types. A value of $p < 0.05$ was defined as significant.

Histochemical procedure for the enzyme assay

After the animal was killed by double pithing, the gastrocnemius muscles were removed immediately. Cross segments (10 mm in length) were then cut longitudinally from the muscle, placed on cork and immediately frozen in liquid N_2-cooled isopentane. Serial transverse sections (10 μm) were cut on a Cryocut E (Reichert Jung, Germany) at –20°C and processed for mATPase activity after acid and alkaline preincubation following a procedure established for amphibian skeletal muscle (Lutz et al. 1998b). For acid inhibition of mATPase activity, sections were preincubated for 3 min in 200 mmol/l sodium acetate (pH 4.75–4.85). Alkaline inhibition consisted of a 15 min preincubation in (in mmol/l) 75 sodium barbitone and 100 $CaCl_2$ (pH 10.05–10.15). After preincubation, the sections were incubated in a solution (in mmol/l): 2.8 ATP, 75 sodium barbitone and 100 $CaCl_2$ (pH 9.4) for either 45 min (acid) or 15 min (alkaline). Slides were washed in three changes of 1% $CaCl_2$ (5 min each) and transferred to fresh 2% $CoCl_2$ for 3 min, followed by six rinses of 0.2% sodium barbitone (30 s each) and four rinses in double-distilled water. Slides were then placed in freshly prepared 0.1% ammonium sulfide for 30 s, washed in water (15 min), and fixed in a 1 : 3 mixture of acetic acid and absolute ethanol (15 min) prior to mounting in glycerol-jelly.

Another 10 μm-thick transverse sections were also cut and incubated for the demonstration of SDH activity (Sieck et al. 1986; Nakatani et al. 2000). Four sections were placed in one staining jar maintained at room temperature (20 ℃). The incubation medium contained (in mmol/l): 80 succinate disodium salt, 1.1 nitroblue tetrazolium, 0.9 sodium azide, and 0.2 1-methoxyphenazine methosulfate in 0.1 mol/l phosphate buffer (pH 7.6) solution. Similar incubating medium was added to a second staining jar containing another four serial sections, except that the substrate for the enzyme reaction, succinate, was omitted. The latter served as control reaction for non-specific staining. The reactions were stopped by thoroughly rinsing the sections with double-distilled water. The sections were post-fixed in 4 % neutral formaldehyde, washed in two changes of water and mounted in glycerol-jelly. The control sections did not show any non-specific staining.

The sections stained for the two enzymes were photographed at magnification $\times$125 and $\times$250 (light microscope Jenaval, Germany) with Kodac 100 Tmax black and white film.

Chemicals used

NaCl, $CoCl_2 \cdot 6H_2O$, sucrose, isopentane (2-methylbutane), succinate disodium salt, sodium barbiturate (CH-9470 Buchs, Fluka AG); KCl, $CaCl_2 \cdot 2\ H_2O$, NaH_2PO_4, Na_2HPO_4, KH_2PO_4, NaOH, sodium acetate (Merck, Darmstadt); adenosine-5'-triphosphate-disodium salt (Serva Feinbiochemica, Heidelberg/New York); sodium azide, 1-methoxyphenazine methosulfate, nitroblue tetrazolium, acetic acid, absolute ethanol, formaldehyde solution, ammonium sulfide solution (Sigma-Aldrich Chemie GmbH).

Results

Electrophysiological characteristic of both muscle fibre types

The duration of ET was significantly shorter in FMFs than in SMFs in both experimental series: for recording of muscle electrical activity, ICAPs (39.66 $\pm$ 7.80 and 154.04 $\pm$ 8.63 s, respectively), and for recording of muscle mechanical activity, twitch (31.47 $\pm$ 3.71 and 142.66 $\pm$ 7.14 s, respectively).

Fatigue-induced changes in characteristic ICAP parameters

The mean absolute parameter values of the first ICAPs in the trials (Table 1) were not statistically different between the fibre types except for LP. Most probably this is due to the influence of hypertonic sucrose bathing medium on the potential parameters and T-tubular anatomy (Khan and Bengtsson 1985; Radicheva et al. 1986; Chawla et al. 2001).

The effect of fatigue on muscle fibre electrical activity (Fig. 2) was expressed in decreased A and NAP values as well as in prolonged LP and ICAP depolarization phase (increased Rt duration). The normalized changes of A and Rt (ΔA and ΔRt) in FMFs were significantly lower than those in SMFs. The difference between LP

Table 1. Absolute initial values of intracellular action potential (ICAP) parameters of slow (SMF) and fast (FMF) fatigable muscle fibres

Fibre type	ICAP parameters				
	A (mV)	NAP (mV)	NAP/A	Rt (ms)	LP (ms)
SMF	97.30 ± 5.37	13.56 ± 3.02	0.162 ± 0.03	0.98 ± 0.08	1.76 ± 0.06
FMF	83.96 ± 4.32	16.14 ± 2.66	0.185 ± 0.02	1.08 ± 0.08	1.16 ± 0.21
p	0.067 n.s.	0.551 n.s.	0.549 n.s.	0.38 n.s.	0.041 *

Values are mean ± SEM ($n = 11$); * $p < 0.05$ – indicates significant difference between the values for the fibre types; A, amplitude of the potential; NAP, amplitude of the negative afterpotential; Rt, rising time; LP, latency period; n.s., non significant.

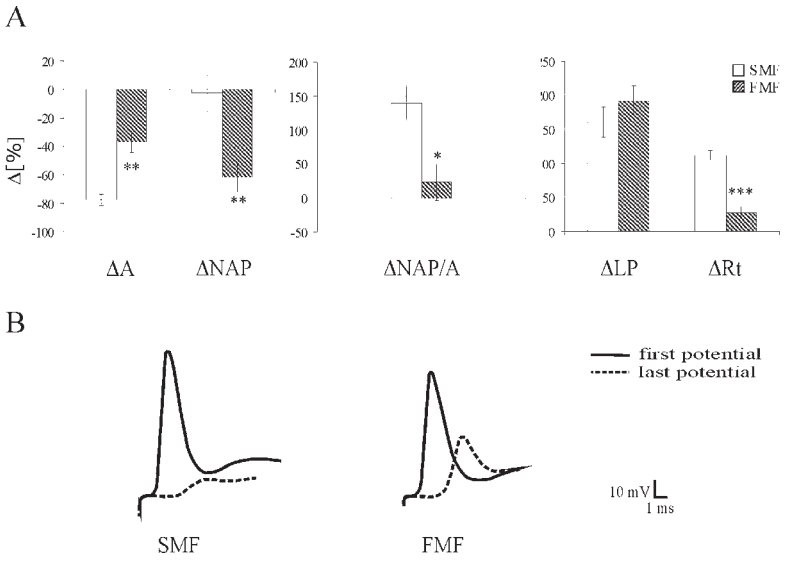

Figure 2. A. Averaged normalized changes (Δ[%]) of intracellular action potential (ICAP) parameters of slow fatigable muscle fibres (SMF) and fast fatigable muscle fibres (FMF) during the period of uninterrupted activity. Values are mean ± SEM ($n = 11$). Asterisks indicate significant differences (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) between the values for the fibre types. **B.** Examples of ICAPs from SMF and FMF at the beginning (first potential, solid lines) and at the end (last potential, dashed lines) of their period of uninterrupted activity (152.56 and 40.22 s for SMF and FMF, respectively).

Table 2. Absolute initial values of mechanical contraction (twitch) parameters of slow (SMF) and fast (FMF) fatigable muscle fibres

Fibre type	Twitch parameters				
	TwF (mN)	CT (s)	HRT (s)	TwF/CT (mN/s)	TwF/HRT (mN/s)
SMF	0.405 ± 0.068	0.063 ± 0.001	0.032 ± 0.002	5.910 ± 1.002	9.474 ± 1.629
FMF	0.855 ± 0.096	0.063 ± 0.002	0.028 ± 0.001	14.327 ± 1.473	26.161 ± 3.239
p	0.001 **	0.879 n.s.	0.025 *	0.00015 ***	0.00042 ***

Values are mean ± SEM ($n = 26$); * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ – indicate significant differences between the values for the fibre types; TwF, twitch force; CT, contraction time; HRT, half relaxation time; TwF/CT and TwF/HRT, average rates of the twitch force rise and decline, respecively; n.s., non significant.

changes in both fibre types was not statistically significant. The mean NAP value of FMFs decreased significantly faster than that of SMFs. The considerable increase in NAP/A ratio in SMFs (ΔNAP/A) was due to the significantly larger decrease in A compared to that in NAP. In contrast, in FMFs the decrease in NAP exceeded that in A, which resulted in smaller increase in their ratio.

Fatigue-induced changes in characteristic twitch parameters

The mean absolute parameter values of the first mechanical contraction during the trials are presented in Table 2. The averaged TwF of FMFs was significantly higher than that of SMFs. The duration of HRT was also significantly different between the fibre types, in contrast to the CT durations. he rates of TwF rise and decline (TwF/CT and TwF/HRT, respectively), in other words, the velocities of both fibre contraction and relaxation were significantly higher in FMFs than in SMFs.

The effect of fatigue on muscle fibre mechanical activity (Fig. 3) was expressed in decreased twitch TwF, CT, TwF/CT and TwF/HRT, as well as in prolonged HRT. The normalized changes in FMFs parameters were significantly lower than those calculated in SMFs except for HRT.

Histochemical investigation of muscle fibres

Identification of fibre types

Based on their relative mATPase reactivity, the fibres of the frog (*Rana ridibunda*) gastrocnemius muscle were classified as one of five different types (1-2, 2, 2-3, 3 and tonic) according to previously established nomenclature for amphibian skeletal muscle (Lutz et al. 1998a). An example for the mATPase reactivity of the fibre types is shown in superficial (Fig. 4A) and deep (Fig. 4B) regions of the muscle.

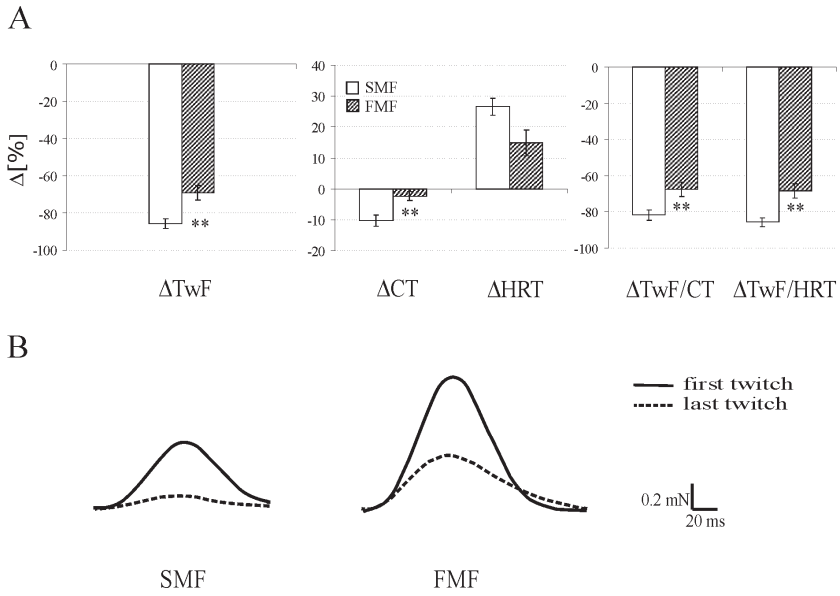

Figure 3. A. Averaged normalized changes ($\Delta[\%]$) of twitch parameters of slow fatigable muscle fibres (SMF) and fast fatigable muscle fibres (FMF) during the period of uninterrupted activity. Values are mean ± SEM ($n = 26$). Asterisks indicate significant differences ($p < 0.01$) between the values for the fibre types. **B.** Twitch examples from SMF and FMF at the beginning (first twitch, solid lines) and at the end (last twitch, dashed lines) of their period of uninterrupted activity (104.46 and 21.83 s for SMF and FMF, respectively).

Under acid preincubation conditions, the smaller tonic fibre demonstrated significant mATPase activity (positive staining), whereas the activity of types 2 (larger sized) and 3 fibres was completely inhibited (negative staining). Larger sized fibres with intermediate mATPase reactivity were classified as type 1-2.

Under alkaline preincubation conditions, types 1-2 and 2 fibres demonstrated significant mATPase activity while the tonic and type 3 fibres were inhibited. Fibres with reactions similar to type 2 but with smaller size we classified as type 2-3. In the processed sections we did not identify pure type 1 fibres, which are expected to demonstrate significant mATPase activity under acid preincubation conditions but under alkaline preincubation conditions should be completely inhibited.

The distribution of the fibre types in the frog gastrocnemius muscle was quantified using mATPase activity under alkaline preincubation conditions. The muscle contained 66.5% fibres with significant mATPase activity (including types 1-2, 2 and 2-3) and 33.5% fibres with inhibited reactivity (tonic and type 3). It should be considered that the fibre distribution was determined only in sections cut from the middle part along the muscle length (the thickest part of the muscle). In these sections, type 1 fibres were not found, probably due to the specific architectonic

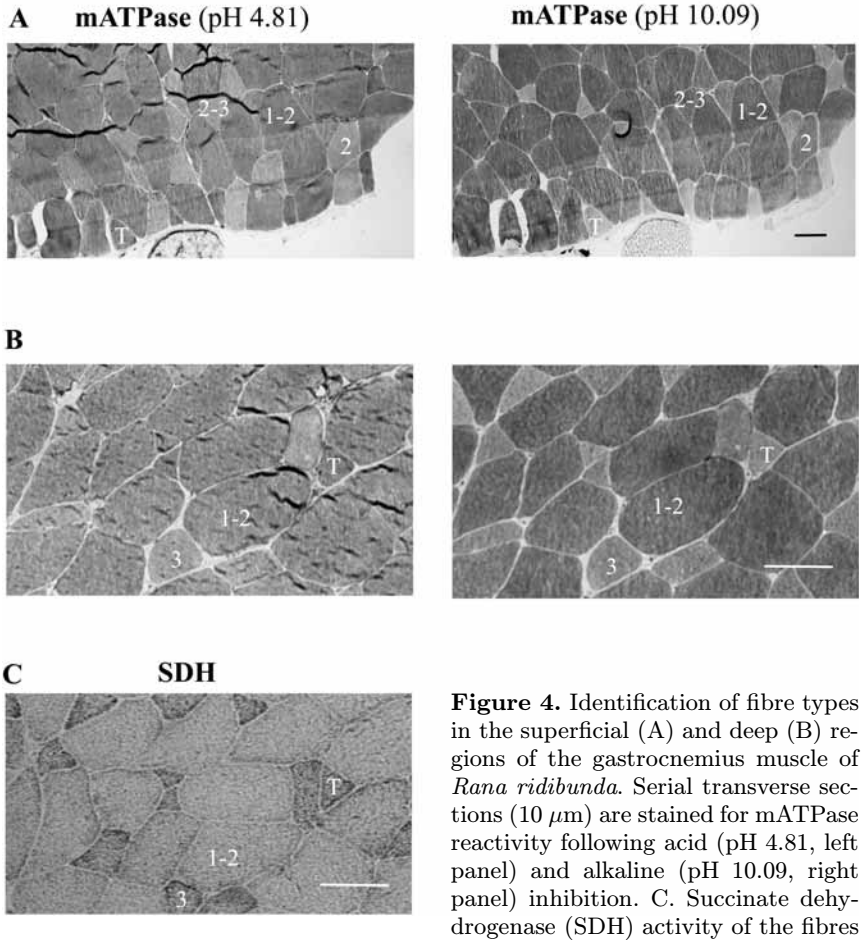

Figure 4. Identification of fibre types in the superficial (A) and deep (B) regions of the gastrocnemius muscle of *Rana ridibunda*. Serial transverse sections (10 μm) are stained for mATPase reactivity following acid (pH 4.81, left panel) and alkaline (pH 10.09, right panel) inhibition. C. Succinate dehydrogenase (SDH) activity of the fibres in the same region as in B. Selected examples of type 1-2, type 2, type 2-3, type 3 and tonic (T) fibres are labelled according to the scheme proposed by Lutz et al. (1998b). Scale bar indicates 100 μm, as the magnification in A is twice as less as in B and C.

structure of the studied muscle (oblique fibres). It is also known that there are differences in fibre type distribution along the muscle length which could arise from sampling of different fibre populations or from differences in myosin isoforms along the length of individual fibres (Edman et al. 1988; Lutz et al. 1998a).

SDH activities of muscle fibres

SDH activities of muscle fibres from the studied muscle are presented in Fig. 4C. The region on the photograph corresponds to that presented in Fig. 4B. Smaller sized fibres (i.e. type 3 and tonic fibres) demonstrated higher SDH activity than that of larger sized fibres (i.e. type 1-2). The subsarcolemal SDH reactivity was

higher than that in the middle of the fibre which corroborates the observations of other studies (Sieck et al. 1986; Martin and Edgerton 1992; Nakatani et al. 2000).

Discussion

In this study we examined the influence of fatiguing stimulation on electrical (presented as ICAPs) and mechanical activity (twitch) of different frog gastrocnemius muscle fibre types. The normalized changes of ICAPs and twitch amplitude and time parameters for the corresponding period of muscle fibre uninterrupted activity were used for evaluation of the fatiguing process. mATPase histochemistry was used in order to validate the fibre types classification based on electrophysiological criteria.

The performed histochemical reaction revealed fibres with different mATPase and SDH activities in the studied muscle. Histochemical, immunohistochemical and morphological evidence has previously confirmed that limb muscles of ranid frogs are composed of one tonic and three twitch fibre types 1, 2 and 3 (Spurway and Rowlerson 1989; Lutz et al. 1998a,b). The fibre size, maximal shortening velocity and mechanical power capabilities of muscle fibre types in Ranid frogs are in order: $1 > 2 > 3 >$ tonic (Lutz et al. 1998a). In the fastest type 1 fibres (high acid-stable mATPase activity), a prevalent glycolytic capacity over the oxidative was found by quantitative histochemical analysis and these fibres should be fast fatigable (Spurway and Rowlerson 1989). Higher oxidative capacity (SDH activity) was evident in the rest of the fibre types and thus they were more fatigue resistant. Lutz et al. (1998a) demonstrated that the large frog muscles, designed to produce significant power during the jump, were composed predominantly of type 1 fibres. In our study we have not identified pure type 1 fibres, but the investigated muscle was composed largely of intermediate type 1-2 fibres in addition to type 2 fibres. The explanation of this finding could be the gastrocnemius muscle function (designed both for jumping and posture support) and/or the specific architecture of the muscle. High levels of co-expression of multiple MHCs, such as type 1-2 and type 2-3, has been found in muscle fibres of different species (Larson and Mos 1993; Peuker and Pette 1997; Lutz et al. 1998a,b; Caiozzo et al. 2003; Medler et al. 2004). Functionally, this co-expression may result in a spectrum of contractile properties exceedingly broader than that defined by the presence of pure MHC isoforms. Despite the variety of muscle fibres shown histochemically, only two types of muscle fibres (SMF and FMF) were distinguished electrophysiologically. They exhibited different mean values of twitch parameters at the beginning of the trial: the TwF, TwF/CT and TwF/HRT (Table 2) in FMFs were significantly higher than in SMFs.

The fatiguing stimulation provoked more pronounced parameter changes in SMFs than in FMFs, which demonstrated their different fatigability. A direct correlation between the fatigue resistance of MU and the oxidative capacity of their constituent muscle fibres has been suggested (Kugelberg and Lindegren 1979; Nemeth et al. 1981; Sieck et al. 1986). In our study, smaller sized fibres (i.e. type 3 and tonic fibres) had higher SDH activity than larger sized fibres (i.e. type 1-2). It is

considered that the cross-sectional areas of high-oxidative muscle fibres are smaller than the areas of low-oxidative muscle fibres because the supplies of oxygen and nutrients for the oxidative energy metabolism from capillaries, which are located close to the membrane, are more plentiful in smaller-sized muscle fibres. An inverse relationship between mean cross-sectional area and mean SDH activity of different types of muscle fibres was observed (Rivero et al. 1998, 1999; Nakatani et al. 2000).

The fatigue induced by the described stimulation protocol could be classified as a high frequency fatigue considering the preparation (frog muscle fibres), the temperature (20–21 ℃) of the medium in the experimental chamber and the short (few seconds to a minute after the end of the trial) recovery periods observed (Radicheva et al. 1998). The stimulation frequency applied in our protocol (5 Hz) is the lowest in the high frequency stimulation range, which allows for a correct evaluation of twitch time parameters. Lower intramuscular microstimulation frequency inducing MU activity guarantees more precise measurement of twitch time parameters (Kossev et al. 1994). In high frequency fatigue, the ionic processes are predominantly affected (Westerblad et al. 1991) in contrast to the low frequency fatigue, where the metabolic exhaustion is involved. According to Westerblad and co-workers (1991), the dominant cause of high frequency fatigue is the impaired propagation of the action potentials into the T-tubules where the shift of ion gradients during intensive activation may be larger. The transport mechanism of the Na^+-K^+ pump of T-tubules membrane may be decisive for the maintenance of excitability (Clausen 2003). The membrane excitability is responsible for the specific pattern of muscle fibre activity observed during long-lasting (fatiguing) stimulation: the period of uninterrupted activity is followed by failures (i.e. inhibition of excitation) and reappearance of the action potentials in different time sequences (Mileva and Radicheva 1996; Radicheva et al. 1998). The observed temporal changes in amplitude, time and spectral characteristics of extracellular action potential (ECAP) from isolated frog muscle fibres repetitively stimulated (Mileva et al. 1998; Vydevska-Chichova et al. 2003) are closely related to the findings in the present study.

The changes in fibre excitation (decrease in amplitude and increase in potential and twitch duration) were observed within a few seconds or minutes depending on the fibre type. During the longer ET of SMFs than that of FMFs, more pronounced changes in their ICAP and twitch parameters were observed.

According to many studies there are some differences in the ionic balance of the different mammalian fibre types (Everts and Clausen 1992; Cairns et al. 1997). An accelerated development of membrane depolarisation in the fast muscle fibres and thus their shorter ET may result from the larger excitation-induced ionic fluxes (Juel 1986) and from the lower sensitivity of Na^+-K^+ pump to increased intracellular Na^+ (Clausen 2003).

Na^+ depletion will reduce the peak of the action potential, which will reduce the Ca^{2+} release from the sarcoplasmic reticulum (SR) especially in the central regions of the muscle fibre (Westerblad et al. 1990). In the present study, the effect of fatigue on muscle fibre mechanical activity was expressed in decreased TwF and CT as well as in prolonged relaxation phase. We found also a significant delay of

TwF/CT and TwF/HRT. The fatigue is generally accompanied by a slowing of relaxation, which could be due to the ATP-dependent SR Ca^{2+} pump working more slowly (Dawson et al. 1980). A decrease in Ca^{2+}-uptake and in Ca^{2+} ATPase activity as well as slowing of the dissociation of force-generating myosin cross-bridges was observed in fatigued muscles (Williams et al. 1998; Inashima et al. 2003).

In conclusion, the performed histochemical reactions revealed that the studied frog gastrocnemius muscle contains fibres with different mATPase and SDH activities. In the electrophysiological experiments, two muscle fibre types conditionally named slow and fast fatigable were distinguished on the basis of their activity pattern characterised by considerably different ET as well as by different dynamics and extent of parameter changes. This suggests that fibre-type dependent characteristic features of fatigue are based on differences in muscle fibre membrane properties, different mATPase isoforms as well as on differences in their oxidative capacity.

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