

Expression of Dihydropyridine and Ryanodine Receptors in Type IIA Fibers of Rat Skeletal Muscle

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In this study, the fiber type specificity of dihydropyridine receptors (DHPRs) and ryanodine receptors (RyRs) in different rat limb muscles was investigated. Western blot and histochemical analyses provided for the first time evidence that the expression of both receptors correlates to a specific myosin heavy chain (MHC) composition. We observed a significant ($p=0.01$) correlation between DHP as well as Ry receptor density and the expression of MHC Ila (correlation factor $r=0.674$ and $r=0.645$, respectively) in one slow-twitch, postural muscle (m. soleus), one mixed, fast-twitch muscle (m. gastrocnemius) and two fast-twitch muscles (m. rectus femoris, m. extensor digitorum longus). The highest DHP and Ry receptor density was found in the white part of m. rectus femoris (0.058 ± 0.0060 and 0.057 ± 0.0158 ODu, respectively). As expected, the highest relative percentage of MHC Ila was also found in the white part of m. rectus femoris ($70.0\pm 7.77\%$). Furthermore, histochemical experiments revealed that the IIA fibers stained most strongly for the fluorophore-conjugated receptor blockers. Our data clearly suggest that the expression of DHPRs and RyRs follows a fiber type-specific pattern, indicating an important role for these proteins in the maintenance of an effective Ca^{2+} cycle in the fast contracting fiber type IIA.

Key words: excitation-contraction coupling, fiber type, myosin heavy chain

I. Introduction

It has long been recognized that Ca^{2+} are essential for muscle contraction [20]. Dihydropyridine receptors (DHPRs) and ryanodine receptors (RyRs) play a crucial role in the calcium signaling process. Some previous studies indicate that these receptors involved in calcium regulation are not equally distributed in the fibers of skeletal muscle. The expression seems to be higher in muscles with a large portion of fast fiber types [13, 17, 30]. Correlation studies between different fast contracting muscle fiber types and the expression of DHPRs and RyRs are still, however, few in number. This study was designed to investigate the expression of DHPR and RyR proteins in different fiber types of mammalian skeletal muscle in more detail.

Skeletal muscle fibers are divided into slow contracting

(type I) and fast contracting (type II) fibers. Fast contracting fibers are further divided into subtypes [31]. Fiber type IIA containing myosin heavy chain (MHC) Ila is the slowest of the fast contracting fiber types and it mainly uses oxidative reactions in energy production. Myoglobin concentration, oxidative enzyme activities and the number of mitochondria are thus high [5, 8]. Fiber type IIB containing MHC IIB is the fastest fiber type, having the highest ATPase activity, but it relies mainly on glycolytic metabolism, and is thus more easily fatigued. Fiber type IID/X containing MHC IID/x has properties between types IIA and IIB [5, 8, 31]. The MHC isoform type has been found to correlate with the contraction velocity of the fiber [31]. Physiologically, skeletal muscles are also divided into two subtypes—fast and slow, according to their different speeds of contraction [36]. Fiber type composition directly reflects the characteristics of the muscle [41].

In all fiber types of skeletal muscle the release of Ca^{2+} from the sarcoplasmic reticulum (SR) is mediated via two membrane proteins: voltage gated calcium channels

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(VGCC) found in T-tubules, and ryanodine receptors (RyRs) in SR [10, 22, 34, 39].

In skeletal muscle, contraction is initiated by depolarization of T-tubules, leading to conformation change of DHPR, the pore-forming α_1 subunit of VGCC. As a consequence, RyR opens and Ca^{2+} flow into the cytoplasm, initiating muscle contraction. In cardiac muscle, on the other hand, muscle contraction occurs via calcium-induced calcium release (CICR). In CICR, Ca^{2+} flow from the extracellular space through the opened DHPR into the cell and mediate calcium release from SR by binding to the RyRs. As a consequence of different Ca^{2+} release mechanism, the time of Ca^{2+} release differs as well, being much longer in cardiac muscle [10, 20]. However, it has been speculated that CICR could be part of the EC coupling cascade in slow twitch skeletal muscle, since there are cardiac isoforms of DHPR in slow muscles as well. This may be related to the substantially slower contraction rate of slow-twitch muscles [10].

Efficient calcium handling capacity is a prerequisite for high contraction velocity. It has been shown that faster contracting fiber types contain more DHPR α_{1S} and RyR1 receptors [13, 17, 30]. Furthermore, the cardiac isoform of DHPR is expressed in slower contracting muscles. The calcium handling capacity seems thus to be somewhat more effective in type II muscle fibers [6]. Moreover, our previous studies indicate that DHPR α_{1S} expression is high in fast oxidative glycolytic (FOG) fiber type [23]. Furthermore, the amount of DHPR correlates with the contraction force of the muscle. Muscles containing a higher percentage of fiber type IIA have larger attenuation of the contraction force as a response to DHPR blocker [24].

The purpose of this study was to examine whether there are differences between muscles with different fiber-type composition at protein level in DHPR and RyR expression. Despite the central role of DHPR and RyR in EC coupling and the clear variety of properties between muscle fibers, no attention has been focused on whether the differences between fibers are also detectable in the expression level of these channel proteins. In the present study, we are going to report the differences of the expression of DHPR and RyR proteins in rat muscles of different types, including one slow-twitch postural flexor (musculus soleus: SO), one mixed fast-twitch flexor (musculus gastrocnemius: GAS), and two fast-twitch extensors (musculus rectus femoris: RF and musculus extensor digitorum longus: EDL).

II. Materials and Methods

Six adult rats (8 mo old, male, Sprague Dawley) weighing 486 ± 22 g (mean \pm SE) were maintained at 21°C with a 12:12-hr light-dark cycle. Food and water were provided ad libitum. The rats were anesthetized with carbon dioxide and decapitated. GAS, RF, SO and EDL were removed, frozen with liquid nitrogen and stored at -80°C .

SDS-PAGE and Western blotting

The muscles GAS and RF were cut into parts contain-

ing red and white sections of the muscle. All muscles were homogenized in 6 vol homogenization buffer (62.5 mM Tris-HCl, pH 6.8) and boiled in sample buffer for 7 min according to Laemmli [19]. Polyacrylamide gel electrophoresis in the presence of SDS (SDS-PAGE) was performed in a Mini-PROTEAN® II dual slab cell system (Bio-Rad) using 5% separating gel for myosin heavy chain isoform typing and 7.5% separating gel for Western blotting. The total protein amount in the samples was 6.5 μg for the separation of MHCs and 65 μg for Western blotting, determined according to Bradford [2]. MHC electrophoresis was performed using 180 V for 3 hr. The gels were stained using Coomassie Brilliant Blue dye. For Western blotting the samples were electrophoresed at a constant voltage of 150 V for one hour. The separated proteins were electroblotted (Mini Trans-Blot Cell, Bio-Rad) to nitrocellulose membrane according to Towbin *et al.* [38], blocked with 5% fat free milk in TBS, rinsed briefly in TBST, and incubated for two hours with primary antibody (L-type Ca^{++} CP α_{1S} , Santa Cruz Biotechnology Inc., U.S.A.; Monoclonal Anti-Ryanodine receptor Clone 34 C, Sigma-Aldrich Inc., U.S.A.), and then with secondary antibody (Blotting Grade Affinity Purified Goat Anti-Mouse IgG H+L Alkaline Phosphatase Conjugate, Bio-Rad), also for two hours. Antibody detection was performed with BCIP/NBT substrate. The dilution for the primary antibody was 1:500 for DHPRs and 1:5000 for RyRs. The optical densities of the detected bands were analyzed with the FluorS MultiImager program (Bio-Rad, U.S.A.).

Fluorescence labeling

Frozen muscle samples were cut into 8 μm cryosections and incubated in 20 nM high affinity (–)-enantiomer of dihydropyridine labeled with orange fluorophore, and 0.5 μM high affinity (–)-enantiomer of ryanodine labeled with green fluorophore (Molecular Probes, Netherlands) for 90 min and processed as described by Mänttari *et al.* [23]. The control samples were preincubated for 10 min in 10 μM DHPR blocker nifedipine and 50 μM RyR blocker dandrolene prior to addition of labeling solution. Images of the sections were obtained with confocal laser scanning microscope (LSM-5 Pascal, Zeiss, Germany) by using excitation at 543 nm for DHPR and 488 nm for RyR.

Fiber typing

Serial 8 μm cross-sections were cut on a cryostat microtome, mounted on cover slips and stained for myosin ATPase according to Brooke and Kaiser [3] (preincubation pH 4.3 and 4.6) and Guth and Samaha [14] (preincubation pH 10.4) and for succinic dehydrogenase (SDH) according to Nachlas *et al.* [26]. The sections were scanned with confocal microscope (LSM-5 Pascal, Zeiss, Germany). The fibers were typed according to their staining in different treatments [3, 14, 16, 27].

Statistical analysis

The correlation between the percentage of fiber types and the relative amount of both DHPR and RyR in all mus-

cles investigated was evaluated with Pearson's correlation test. The statistical analysis was performed with SPSS 12.0 for Windows. A p value of <0.05 was accepted as indicator of a significant correlation.

III. Results

In order to characterize the amount of DHPR and RyR in different muscles, electrophoresis and Western blotting methods were used. The relative amounts of DHPRs and RyRs in all the muscles investigated are presented in Figure 1. The highest DHPR and RyR optical densities were found in the white part of RF (0.058±0.0060 ODu and 0.057±0.0158 ODu, respectively). The lowest densities were found in the red part of RF (0.008±0.0017 ODu and 0.018±0.0017 ODu, respectively).

To compare the DHPR and RyR densities with the fiber type composition in different muscles, myosin heavy chain analysis was performed for each muscle studied. The results from the separation of MHCs are shown in Figure 2. The highest relative percentage of MHC IIa was found in the white part of RF (70.0±7.77%). There was also a significant (p=0.01) positive correlation between DHPR as well as RyR density and the expression of MHC IIa (Pearson's correlation factor $r=0.674$ and $r=0.645$, respectively). Analysis without top right data point between RyR and MHCIIa correlation resulted also in significant relationship (p=0.02). The results of the correlation analysis are presented in Figure 3. The density of neither receptor correlated significantly

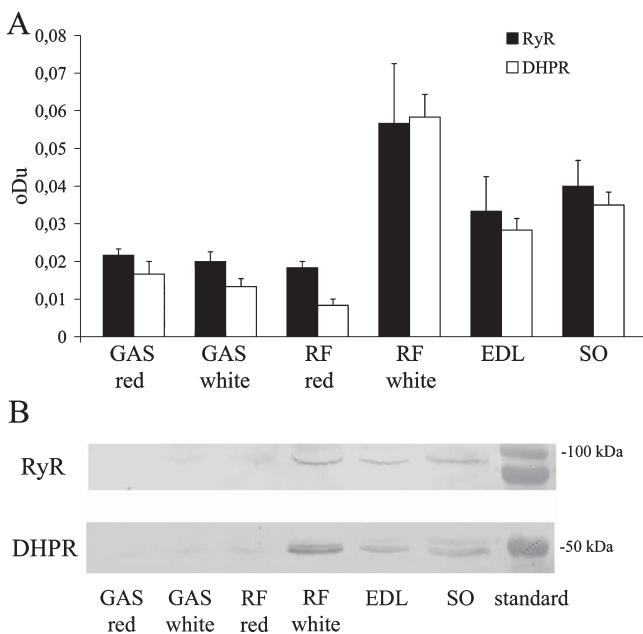


Fig. 1. (A) Optical density unit (oDu) values of ryanodine receptors (RyRs) and dihydropyridine receptors (DHPRs) in red and white parts of musculus gastrocnemius (GAS) and musculus rectus femoris (RF) and in musculus extensor digitorum longus (EDL) and musculus soleus (SO) (n=6 per muscle). (B) Representative graph of RyRs and DHPRs in skeletal muscle homogenates.

with any other MHC types.

Fluorescence labeling and fiber typing

Figure 4 shows the localization of DHPR and RyR in different hind leg muscles of rat (two upper panels, respectively). Both receptors were moderately to highly expressed in IIA skeletal muscle fibers, while only barely expressed in others. In order to characterize the fiber-type specificity of DHPR and RyR, serial sections were assayed for the expression of the receptors concerned, and for ATPase as well as SDH activity. Fiber types were separated from each other by their differential staining in ATPase and SDH treatments. Figure 4 shows serial transverse sections of the muscles studied stained for SDH and for ATPase activity at pHs 4.3, 4.6 and 10.4 (four lower panels, respectively). Preincubation in pH 4.3 resulted in ATPase activity only in fiber type I, staining it dark but leaving all the other fiber types unstained. In pH 4.6 also the fast contracting fibers were stained. Fiber type I had still the highest staining intensity, while fiber types IIB and IID/X were moderately stained. In

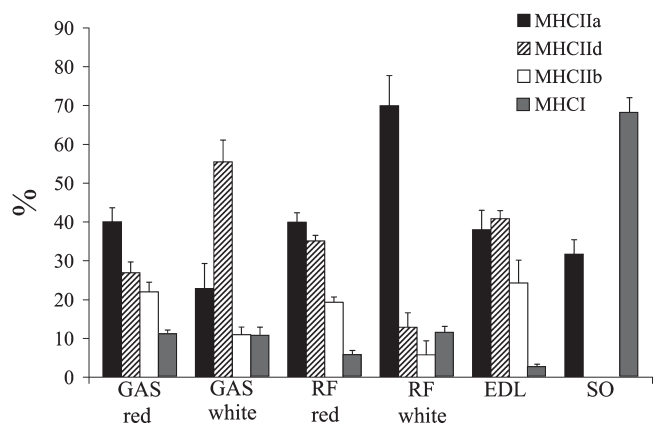


Fig. 2. Relative percentages of different myosin heavy chain (MHC) isoforms in different muscles of rat hind leg.

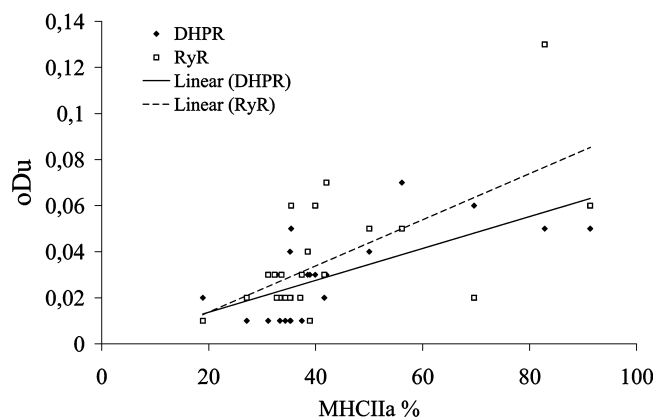


Fig. 3. Regression lines for the optical density unit (oDu) value of receptors, and the percentage of myosin heavy chain (MHC) IIa. Pearson's correlation factor for DHPR: $r=0.674$ and for RyR: $r=0.645$ (p=0.01) (n=6 muscles and 6 samples per muscle).

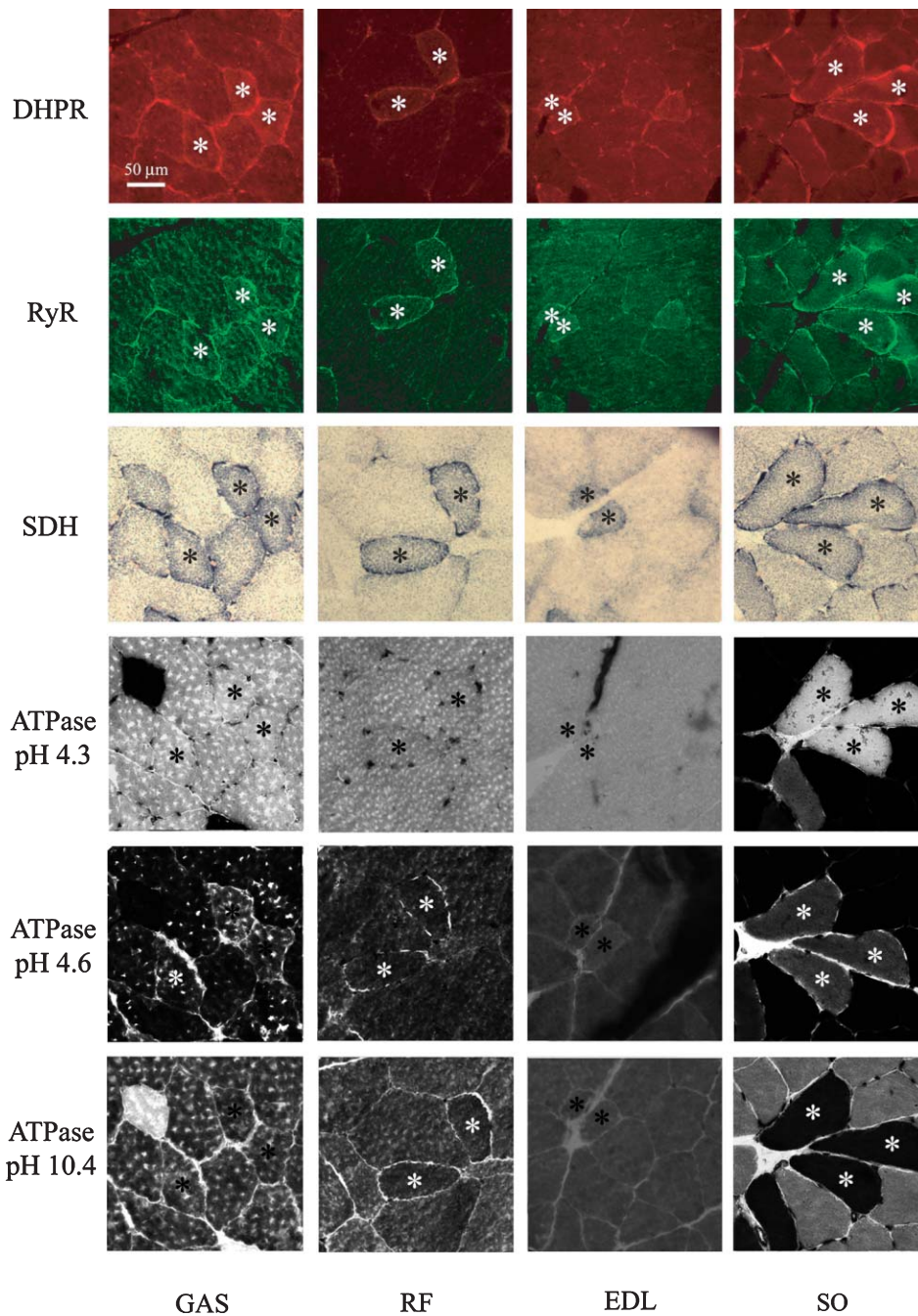


Fig. 4. Serial sections from musculus gastrocnemius (GAS), musculus rectus femoris (RF), musculus extensor digitorum longus (EDL) and musculus soleus (SO). Dihydropyridine receptor (DHPR) and ryanodine receptor (RyR) expression was investigated by incubating muscle sections in 20 nM high affinity (-)-enantiomer of dihydropyridine labeled with orange fluorophore, and 0.5 μ M high affinity (-)-enantiomer of ryanodine labeled with green fluorophore, respectively (two upper panels). Analysis of the muscle fiber types was performed with SDH staining method and with different myosin ATPase (mATPase) preincubation treatments (pH 4.3, 4.6 and 10.4) (four lower panels, respectively). In SDH treatment the highest SDH activity is seen in the fiber type IIA with the most intensive staining. Fiber type I shows also positive reaction while in types IIB and IID/IIX the SDH activity diminishes in the order of IID/IIX>IIB. The preincubation in acid pH 4.3 inhibits the mATPase activity in all the fast contracting fiber types and only type I fibers are stained dark. Fiber type IIA appears pale in preincubation pH 4.6 while type I has the most intensive staining affinity. Fiber type IID/X is stained somewhat stronger than type IIB. The preincubation in alkaline pH 10.4 inhibits the mATPase activity in fiber type I. Therefore the most intensive staining is observed in fiber types IIA and IID/X. Fiber type IIA is marked with asterisk.

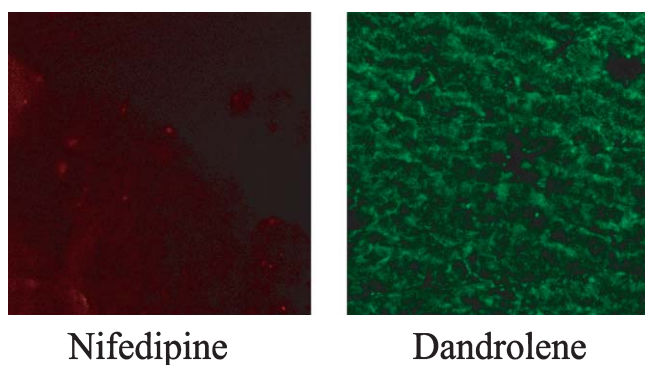


Fig. 5. Control samples preincubated with DHPR blocker nifedipine and RyR blocker dandrolene.

pH 4.6 fiber type IIA had the lowest staining intensity. Contrary to acid preincubation, in pH 10.4 fiber types IIA and IID/X possessed the highest ATPase activities, while the enzyme in type I was inhibited. In pH 10.4 fiber type IIB showed moderate staining. In contrast to other muscles, in m. soleus fiber type I, which in other muscles showed quite high oxidative enzyme activity, was weakly stained after SDH treatment.

According to representing fibers in serial sections, the highest fluorescence intensity, indicating the highest DHPR and RyR density, was observed in fiber type IIA in all the muscles studied. Moreover, the highest intensity was accumulated near the sarcolemma of muscle fibers. The corresponding control samples, preincubated with nifedipine and dandrolene, resulted in a loss of staining (Fig. 5).

IV. Discussion

The main finding in the present study was higher expression of both DHPRs and RyRs in fiber type IIA compared with other fiber types of skeletal muscle. All the experiments performed supported this finding. First, the amount of both receptors correlated significantly with the relative percentage of myosin heavy chain IIA in all muscles investigated (Fig. 3). Second, according to the results of fluorescent microscopy, the somewhat increased receptor density was apparent in fiber type IIA (Fig. 4). Interestingly, it seems that the highest expression of both receptors is continuous near the sarcolemma of fibers. The method used in this study is, however, evaluated to be accurate since in previous investigations using the same staining protocol the receptors in T-tubules inside the fibers are seen both in cross and longitudinal sections [23, 25]. Moreover, the corresponding control slides, preincubated with receptor blockers, resulted in a loss of staining (Fig. 5). In previous studies it has been noted that DHPR is expressed both in T-tubules and in plasma membrane of the muscle cell [12]. Moreover, it has been shown that skeletal muscle fibers contain longitudinal T-tubules beneath the sarcolemma [11]. In this study, mainly the receptors in these tubules and in the plasma membrane are visualized in the cross sections of muscles,

although moderate staining is also seen inside the fibers. Similar results were previously observed in rabbit ventricle and atrium [4].

The fiber type composition of the muscles was quite similar compared to previous studies [1, 8]. The percentage of fast contracting fibers was highest in EDL as has also previously been shown [1, 8]. On the contrary, in the postural muscle SO, the percentage of fiber type I was dominant.

The main aim of this study was to test the hypothesis that DHPR as well as RyR expression is a fiber type specific characteristic. To test this assumption, it was essential to examine a broad range of fiber types, including all four types known to appear in rat hind limb muscles, i.e. types I, IIA, IIB, and IID/X. Skeletal muscle fibers can be classified histologically according to their staining in differential ATPase preincubation pHs and according to their oxidative enzyme activities [16]. In some previous studies the oxidative enzyme activities of fibers were in the rank order of type IIA>type I>type IID/X>type IIB [8]. This is also seen in our results, where fiber type IIA shows the highest succinic dehydrogenase (SDH) activity. However, SO forms an exception. Fiber type I, which has quite positive enzyme activity in other muscles, shows low enzyme activity in the SO muscle after SDH treatment. Similar results have also been seen in prior investigations [27]. In the present study, fiber types were classified according to their staining in different treatments. Since we took serial sections from muscles, the same fiber could be identified from different sections. Based on the fiber type classification, the highest fluorescence intensity was thus localized to fiber type IIA in all muscles investigated.

Fiber type IIA contracts relatively fast and uses mainly oxidative metabolism to produce energy for force generation. Therefore, fiber type IIA is active both at quite low and high intensity locomotion velocities. Moreover, IIA is relatively fatigue resistant and is recruited in long-lasting high intensity events [8]. This sets high demands for the Ca²⁺ handling capacity of the fiber type. Both the release and reuptake of Ca²⁺ must occur fast and continuously. This could be the reason why the amount of channels involved in the Ca²⁺ sequestering system is somewhat higher in fiber type IIA than in other fiber types. Our previous studies on DHPR fiber type specificity [23] revealed corresponding results. It has been shown that the density of DHPR is an important factor influencing the overall contractile properties of the muscle. According to previous results, DHPR expression is higher in muscles containing a relatively high percentage of fiber type IIA [24].

No previous investigations have been made on the expression of RyR in different fast twitch skeletal muscle fiber types. The comparisons are restricted to the level of differentially contracting muscles [13, 30]. In the present study it was, however, observed that also RyR shows strong expression in fiber type IIA. In skeletal muscle, the DHP α_{1S} and Ry1 receptors are directly connected to each other during EC coupling [21, 37]. It thus seems logical that the density of both receptors is high in the same muscle fiber type. The

active use of fiber type IIA could, however, be just one reason for results found in the present study. Previously it has been shown that faster contracting fiber types contain more SR than slower ones [9]. Since RyRs are expressed in SR, the differential level of SR between fiber types could also effect on the result.

To quantify the expression of DHPRs and RyRs in different muscles, Western blotting method was used. During the analysis it was observed that the molecular weights of the receptors were lower than expected. In previous investigations it has, however, been observed that both receptors degrade during handling, resulting in fragments observed also in the present study [15, 18, 29, 40]. In this study, the expression of MCH IIA correlated positively with the level of both DHPR and RyR. On the other hand, there was no correlation between the other MHC types and the level of receptors. This indicates a fiber type IIA specificity of both DHP and Ry receptors. This also suggests that those muscles that contain higher level of MHC IIA have also relatively higher level of the receptors. There are not, however, clear differences in the expression of receptors between other MHC types. In this study it was also observed that the highest DHPR and RyR densities were detected in the white part of RF. The expression of fiber type IIA was also most augmented in the white part of RF. RF is one of the main power-producing muscles during running, being active twice per step cycle at higher running speeds [7, 28]. Though GAS is also a power-producing muscle, it has been shown that it is activated mostly during the highest running velocities [32, 35]. It is thus quite consistent that both the amount of fiber type IIA, and DHPR as well as RyR expression are high in RF as compared to other muscles.

The level of receptors was not same in the whole RF since the red part of RF contained lower level of receptors compared with the white part, as expected. The MHC type composition differs between these two parts of RF affecting the results. Interestingly, in GAS there were no significant differences between the different parts of the muscle. One reason for this could be the limited freedom of movement of laboratory rats in the cages. Receptors are a significant part of the muscle contraction. Postural muscles are active constantly and this is seen in the higher level of receptors in SO when compared to the red part of GAS. Although red GAS contains relatively high level of slow contracting fiber types, it is not active when an animal is standing [28, 33]. The white part of GAS is, on the other hand, used only in the highest running velocities [32, 35]. The restricted area for fast movements could thus be one of the reasons for the observed similarities between the white and red parts of GAS. The correlation between the density of DHPR and RyR and the amount of IIA fiber types still, however, exists also in GAS.

To conclude, this study demonstrates for the first time that both DHPRs and RyRs are somewhat higher expressed in one distinct fiber type of skeletal muscle. According to the results from histochemical and protein analysis, both DHPR and RyR expression is strongest in fiber type IIA in all mus-

cles investigated. Fiber type IIA also showed the highest oxidative enzyme activity. This suggests an important role of effective Ca^{2+} handling in the fast contracting fiber type concerned.

V. References

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