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Analysis of excitation-contraction-coupling components in chronically stimulated canine skeletal muscle

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The chronic stimulation of predominantly fast-twitch mammalian skeletal muscle causes a transformation to physiological characteristics of slow-twitch skeletal muscle. Here, we report the effects of chronic stimulation on the protein components of the sarcoplasmic reticulum and transverse tubular membranes which are directly involved in excitation-contraction coupling. Comparison of protein composition of microsomal fractions from control and chronically stimulated muscle was performed by immunoblot analysis and also by staining with Coomassie blue or the cationic carbocyanine dye Stains-all. Consistent with previous experiments, a greatly reduced density was observed for the fast-twitch isozyme of Ca^{2+}-ATPase, while the expression of the slow-twitch Ca^{2+}-ATPase was found to be greatly enhanced. Components of the sarcolemma (Na^{+}/K^{+}-ATPase, dystrophin-glycoprotein complex) and the free sarcoplasmic reticulum (Ca^{2+}-binding protein sarcalumenin and a 53-kDa glycoprotein) were not affected by chronic stimulation. The relative abundance of calsequestrin was slightly reduced in transformed skeletal muscle. However, the expression of the ryanodine receptor/Ca^{2+}-release channel from junctional sarcoplasmic reticulum and the transverse tubular dihydropyridine-sensitive Ca^{2+} channel, as well as two junctional sarcoplasmic reticulum proteins of 90 kDa and 94 kDa, was greatly suppressed in transformed muscle. Thus, the expression of the major protein components of the triad junction involved in excitation-contraction coupling is suppressed, while the expression of other muscle membrane proteins is not affected in chronically stimulated muscle.

In excitation-contraction (EC) coupling of skeletal muscle, Ca^{2+} release from the sarcoplasmic reticulum (SR) is initiated and coupled to the depolarization of the transverse tubular membrane system [1, 2]. The ryanodine receptor (RyR) of rabbit skeletal muscle has been purified and shown to be identical to the Ca^{2+}-release channel of the SR [3–5]. The 'foot' structure at the junction between the SR and the transverse tubules is composed of the Ca^{2+}-release channel and has been proposed to interact with the transverse tubule tetrad [6]. The receptor for the 1,4-dihydropyridine class of Ca^{2+} blockers (DHPR) functions in skeletal muscle, not only as a Ca^{2+} channel, but also as the voltage sensor in EC coupling [7, 8], and is probably the primary component of the transverse tubule tetrad. The subunit composition of the DHPR from rabbit skeletal muscle has been analyzed [9, 10] and each of the subunits have been cloned [8, 11–13]. Other components found in the triad fraction of skeletal muscle are the junctional sarcoplasmic reticulum (JSR) proteins of apparent 90 kDa and 94 kDa [14], which could play a regulatory role in EC coupling or Ca^{2+} release. In muscle relaxation, the extensive intracellular membrane system, the SR, achieves the rapid reuptake of Ca^{2+} from the cytosol and storage in its lumen. Extensive information has been accumulated about the major components of this organelle, including Ca^{2+}-ATPase and calsequestrin [15, 16].

Based on differences in contractile and ultrastructural parameters, mammalian skeletal muscle may be subdivided into fast-twitch and slow-twitch skeletal muscle [17–19]. A conversion of fast-twitch skeletal muscle into slower-contracting muscles can be induced by chronic, low-frequency stimulation [20]. This functional transformation was most extensively studied with respect to contractile proteins, as reviewed in [21]. Conditioned skeletal muscle has possibly an important medical application in supporting circulation as constructed skeletal muscle ventricles. Chronically stimulated latissimus dorsi was found to have the potential to directly support the circulation [22]. Recently, we could show that chronic stimulation of a predominantly fast-twitch skeletal muscle enhanced the expression of type-I (slow-twitch muscle) Ca^{2+}-ATPase and suppressed the expression of the type-II (fast-twitch muscle) Ca^{2+}-ATPase [23].

The purpose of this investigation was to analyze the expression of marker proteins of the major skeletal muscle membrane systems in chronically stimulated muscle, including important components involved in EC coupling. Unlike the Ca^{2+}-ATPase isoforms in which fast-twitch Ca^{2+}-ATPase is found in fast-twitch muscle and the cardiac Ca^{2+}-ATPase

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Abbreviations. DAG, dystrophin-associated glycoprotein; DHPR, dihydropyridine receptor; EC, excitation/contraction; SR, sarcoplasmic reticulum; JSR, junctional sarcoplasmic reticulum; mAb, monoclonal antibod(y)ies; RyR, ryanodine receptor; CS, calsequestrin; DYS, dystropin; GP, glycoprotein.

isoforms is present in slow-twitch muscle, only the skeletal muscle forms of RyR and DHPR are detectable in fast-twitch and slow-twitch skeletal muscle [24, 25]. Skeletal muscle and cardiac RyR isoforms differ significantly in their primary sequence and are clearly products of different genes [26]. Thus, it is interesting to investigate the fate of these two EC-coupling components in the fast-to-slow-twitch skeletal muscle transition. Our immunoblot analysis of the effect of chronic stimulation on the canine latissimus dorsi included proteins of the longitudinal SR and JSR, the transverse tubules and the sarcolemma. We found that the expression of the RyR and two JSR proteins of 90 kDa and 94 kDa, as well as the transverse tubular DHPR was greatly reduced in chronically stimulated skeletal muscle. In comparison, sarcolemma marker Na/K-ATPase and components of the dystrophin-glycoprotein complex and the Ca^{2+}-binding protein sarcalumenin were not affected in stimulated muscle.

EXPERIMENTAL PROCEDURES

Stimulation of skeletal muscle

Chronic stimulation of canine skeletal muscle was carried out by the method of Mannion et al. [22]. In the dogs studied, the right latissimus dorsi was used as the control, while the experimental muscle on the left side was stimulated, without a vascular delay period, at 2 Hz via the thoracodorsal nerve. The animals underwent a conditioning period for 6–8 weeks [23].

Isolation of the microsomal fraction

A microsomal fraction containing vesicles derived from longitudinal SR and JSR, transverse tubules and sarcolemma was isolated from control canine latissimus dorsi, control vastus intermedius, and conditioned latissimus dorsi as follows. Immediately upon removal from the anesthetized animals the muscle was cooled, the mass determined and homogenized twice for 30 s in an Omnimixer at the maximal speed setting. The volume of homogenization solution was 6.5 times the mass of the muscle. The composition of the homogenization solution was 0.25 M sucrose and 20 mM Mops, pH 7.0, with the following protease inhibitors; aprotinin, 0.5 μg/ml; benzamidine, 100 μg/ml; leupeptin, 0.5 μg/ml; pepstatin A, 0.5 μg/ml; phenylmethylsulfonyl fluoride, 40 μg/ml. The supernatant from the centrifugation of the homogenate at $6000 \times g$ for 15 min was filtered through four layers of cheesecloth and centrifuged at $30000 \times g$ for 120 min. The pellet from this spin was suspended in 0.6 M KCl, 0.25 M sucrose and 20 mM Mops, pH 7.0, with the above protease inhibitors for 30 min before centrifugation at $117000 \times g$ for 30 min. The pellet from this spin, the microsomal fraction, was suspended in 40% sucrose and 20 mM Mops, pH 7.0, with the above protease inhibitors and stored at −70°C until use. Protein concentration was determined as described [27] with bovine serum albumin as standard.

Monoclonal and polyclonal antibodies

Production and characterization of monoclonal antibodies (mAb) against skeletal muscle proteins was carried out by immunizing mice with various rabbit skeletal muscle membrane preparations, as described previously [25, 28, 29]. An immunodot assay was used for the initial screening of the hybridoma supernatants, followed by immunoblot and immunofluorescence analysis [30]. The following monoclonal antibodies were used in this study: mAb IID8 and IIH11 against the slow-twitch and fast-twitch Ca^{2+}-ATPase of sacroplasmic reticulum [29]; mAb VIIID1_2 against calsequestrin; mAb IIID5 against the α_1 subunit of the DHPR [25]; mAb XIIC4 against the 53-kDa SR glycoprotein and sarcalumenin (160 kDa protein) [31]; mAb VIIC12_3 against a 90-kDa protein of JSR; mAb VIA4_2 against dystrophin [32]; mAb VIA4_1 against the dystrophin-associated glycoprotein (DAG) of 156 kDa [32]. mAb McB2 against Na^+/K^+-ATPase [33] and mAb RyR-1/RyR-3 [24] against cardiac RyR were generous gifts from Dr K. Sweadner (Harvard Medical School) and Dr T. Imagawa (Osaka University), respectively.

Polyclonal antisera against the 94-kDa glycoprotein of JSR were raised in guinea pigs. Gel slices of the 94-kDa glyoprotein of JSR (devoid of a contaminating Ca^{2+}-ATPase protein band) were homogenized and used for immunization as described [34]. Production and specificity of antibodies was tested by immunoblot analysis of JSR and microsomes from rabbit skeletal muscle.

Gel electrophoresis and densitometric analysis

SDS/PAGE was performed according to Laemmli [35] in 3–12% polyacrylamide gels. Protein bands were visualized by Coomassie-blue staining and also analyzed by Stains-all staining for Ca^{2+}-binding proteins [36]. Coomassie-blue-stained gels contained 50 μg protein in each lane while Stains-all gels contained 100 μg protein in each lane. Densitometric scanning of Coomassie-blue-stained SDS/polyacrylamide gels was carried out on a Molecular Dynamics 300S computing densitometer and also on a Hoefer Instruments densitometer model GS 300.

Immunoblot analysis

Proteins separated by SDS/PAGE were transferred to nitrocellulose membranes by the method of Towbin et al. [37]. Prestained molecular mass standards were from Bethesda Research Laboratories and their apparent molecular masses were as follows: myosin, 224 kDa; phosphorylase *b*, 109 kDa; albumin, 72 kDa; ovalbumin 46 kDa; carbonic anhydrase, 29 kDa. Nitrocellulose transfers were blocked in Blotto (bovine lactor transfer technique optimizer containing 50 mM sodium phosphate, pH 7.4, 150 mM NaCl and 5% non-fat dried milk) and subsequently incubated overnight with primary antibody (hybridoma supernatant or 1:1000 dilution of a concentrated antibody or antisera). Immunoblots were then washed with Blotto and incubated for 1 h with peroxidase-conjugated secondary antibody (Boehringer-Mannheim) at a dilution of 1:1000. After washing the nitrocellulose blots with Blotto, they were developed in 20 mM Tris/Cl, pH 7.5, and 200 mM NaCl using 4-chloro-1-naphthol as substrate [38]. Immunoblots contained the following amounts of protein in each lane for the different antigens to be tested: Ca^{2+}-ATPase and calsequestrin (20 μg); dystrophin and 156-DAG 150 μg; all other antigens, 100 μg.

RESULTS

Microsomal preparation

This study investigates the effect of chronic stimulation on membrane proteins in canine skeletal muscle. Preliminary

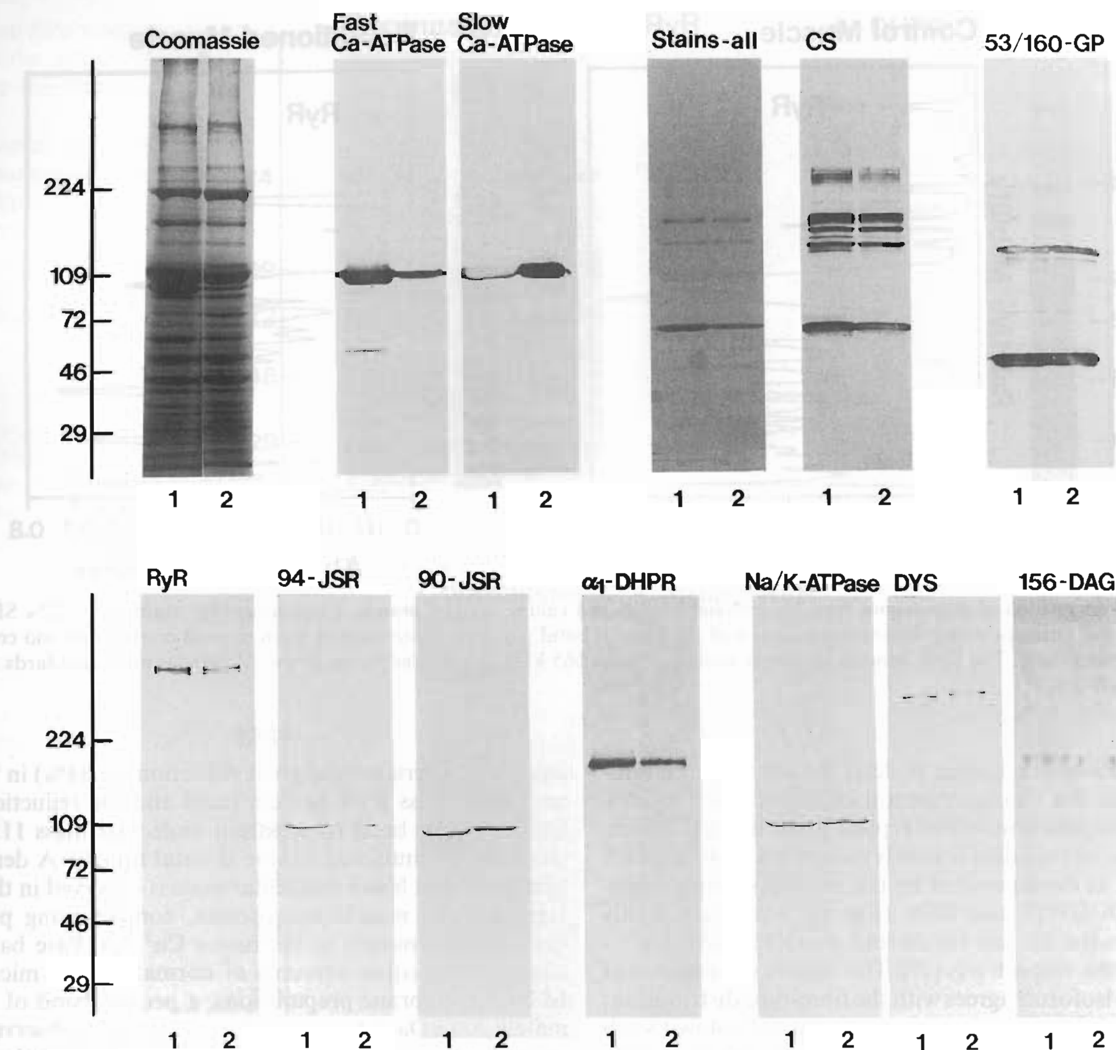

Fig. 1. Immunoblot analysis of microsomal proteins in slow-twitch and fast-twitch canine skeletal muscle. A Coomassie-blue and a Stains-all-stained gel are shown plus identical immunoblots of microsomes from predominantly fast-twitch latissimus dorsi (lane 1) and predominantly slow-twitch vastus intermedius (lane 2) canine skeletal muscle. Immunoblots were stained with mAb IIH11 against fast-twitch SR Ca^{2+}-ATPase (Fast Ca-ATPase), mAb IID8 against slow-twitch SR Ca^{2+}-ATPase (Slow Ca-ATPase), mAb VIIID1$_2$ against calsequestrin (CS), mAb XIIC4 against 160-kDa sarcalumenin and a 53-kDa glyoprotein (53/160-GP), mAb XA7 against RyR, guinea pig polyclonal antisera against JSR protein of 94 kDa (94-JSR), mAb VIIC12$_3$ against JSR protein of 90 kDa (90-JSR), mAb IIID5 against α_1-subunit of DHPR, (α-DHPR) mAb McB2 against Na^+/K^+-ATPase (Na/K-ATPase), mAb VIA4$_2$ against dystrophin (DYS) and mAb VIA4$_1$ against 156-kDa DAG (156-DAG). Molecular mass standards are shown on the left (kDa).

analysis of total extracts from normal control and conditioned muscle indicated that crude muscle extracts were not sufficiently enriched in membrane proteins over highly abundant myofibrillar proteins to be suitable for direct immunological analysis. On the other hand, extensive subcellular fractionation procedures for the isolation of different membrane systems could affect yield and composition of the membrane systems studied. Therefore, we carefully prepared, in the presence of a protease inhibitor cocktail, a crude skeletal muscle microsomal fraction containing longitudinal SR and JSR, transverse tubules and sarcolemma vesicles. A detailed immunoblot analysis of marker proteins which are characteristic for these four different skeletal muscle membrane systems was carried out using the crude microsomal fraction from normal control and conditioned canine skeletal muscle (see Figs 1, 3 and 4). This preparation allows comparison of differences in skeletal muscle membrane composition.

Immunoblot analysis of microsomal proteins in slow-twitch and fast-twitch canine skeletal muscle

To understand better the transition of fast-to-slow-twitch muscle protein isoform expression, the relative distribution of these isoforms normally occurring in fast-twitch and slow-twitch muscle must first be determined. Indirect immunofluorescence microscopy has been used to study the fibre-type distribution of specific isoforms of muscle proteins [25, 29]. However, the antibodies to the EC-coupling proteins investigated in this study were originally produced against rabbit skeletal muscle proteins and did not exhibit strong-enough labeling in immunofluorescence techniques to demonstrate their fibre-type distribution in canine skeletal muscle. Previously, we used extensive immunoblot analysis to determine the distribution of marker proteins in subcellular fractions from rabbit skeletal muscle [30]. We therefore performed a comparative immunoblot analysis of predominantly fast-

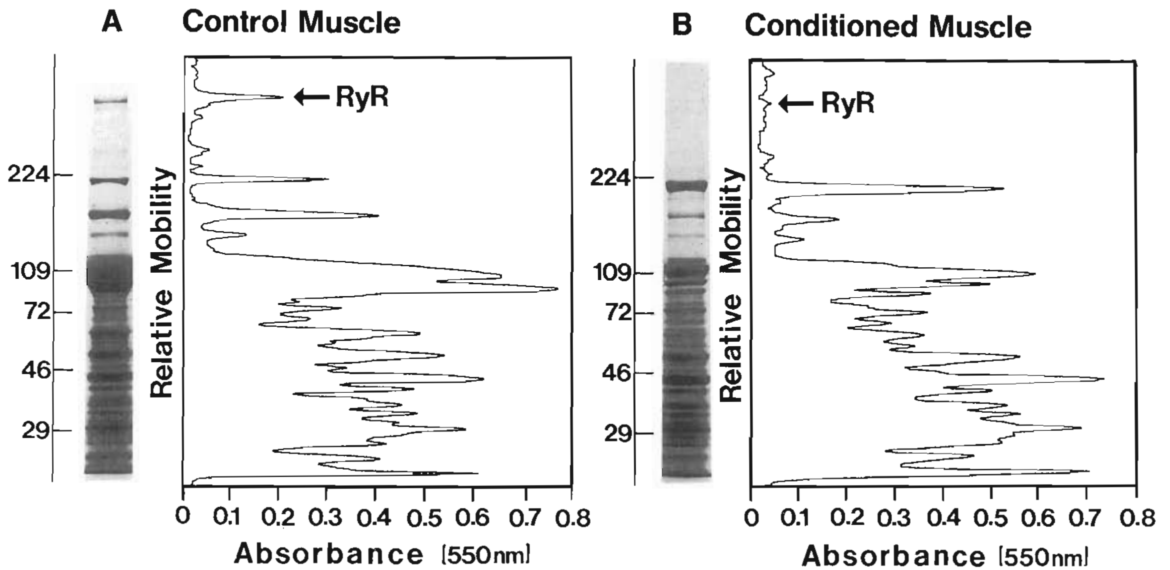

Fig. 2. Protein composition of microsomes from control and conditioned canine skeletal muscle. Coomassie-blue stained 3–12% SDS/PAGE gels are shown and corresponding densitometric scans of the protein band pattern of microsomes from normal control (A) and conditioned (B) canine skeletal muscle. The RyR band of apparent molecular mass 565 kDa is indicated by an arrow. Molecular mass standards are shown on the left of each gel.

twitch and slow-twitch canine skeletal muscle with the antibodies used in the characterization of conditioned muscle samples. Canine latissimus dorsi is predominantly fast-twitch, whereas vastus intermedius is mostly composed of slow-twitch muscle fibres, as demonstrated by the relative staining intensity with mAb IIH11 and IID8 (Fig. 1), which are highly specific probes for the fast-twitch and slow-twitch SR Ca^{2+}-ATPase isoforms, respectively [29]. The relative distribution of Ca^{2+}-ATPase isoforms agrees with the fibre-type distribution; 71% fast-twitch for the latissimus dorsi and 88% slow-twitch for vastus intermedius, as reported by Armstrong et al. [39]. The relative abundance of calsequestrin appears to be slightly reduced in slow-twitch muscle, as demonstrated by Stains-all staining and immunoblot labeling with mAb $VIIID1_2$ (Fig. 1). On the other hand, 160-kDa sarcalumenin and the 53-kDa glycoprotein of SR and sarcolemma marker proteins Na^+/K^+-ATPase, dystrophin and the 156-kDa DAG are equally abundant in both muscle types. Immunoblot analysis of the RyR and the JSR protein of 94 kDa, as well as the α_1-subunit of the DHPR, illustrated a relative lower presence of these components in slow-twitch muscle. Incubation with monoclonal antibodies against the cardiac isoform of the RyR showed that this isoform is not present in slow-twitch skeletal muscle. In comparison to other components of JSR, the JSR 90-kDa protein, was, surprisingly, found to be equally distributed between both fibre types.

Protein composition of microsomes from control and conditioned canine skeletal muscle

The microsomal fractions from normal control and chronically stimulated skeletal muscle were separated by SDS/PAGE on 3–12% gels to compare their protein composition and to characterize various proteins of interest in immunoblotting experiments. Fig. 2 compares the Coomassie-blue-stained SDS/PAGE profile and corresponding densitometric scans of the control and stimulated muscle fractions. The most obvious difference between identifiable protein bands in the complex pattern are the great reduction ($\approx$ 84%) in the high-molecular-mass RyR protein band and the reduction in the Ca^{2+}-ATPase band of apparent molecular mass 115 kDa in chronically stimulated canine skeletal muscle. A denser protein pattern at lower molecular mass is observed in the stimulated skeletal muscle microsomes, compensating partly for the reduced amount of the major Ca^{2+}-ATPase band when compared to equal amounts of normal control microsomes. In both membrane preparations, a protein band of approximately 200 kDa, which is probably myosin, is observed. Myosin and actin content of crude skeletal muscle membranes was very variable. This phenomenon is illustrated in Fig. 3, which compares the Coomassie-blue-stained SDS/PAGE profiles of skeletal muscle membranes from two different experimental dogs. Due to the large abundance of these proteins in muscle tissue, variation in myosin and actin content is a problem of the preparation of skeletal muscle membranes. However, in light of several preparations these discrepancies in the amount of myosin and actin do not change the overall analysis of EC-coupling components in conditioned sleketal muscle. Previous studies established that chronic low-frequency stimulation affects the expression of fast-twitch myosin heavy-chain isoforms. Pette and co-workers could show that chronic stimulation of rabbit skeletal muscle leads to increases in myosin heavy-chain isoform IIa at the expense of myosin heavy-chain isoform IIb [40–42]. Volume integration on a Molecular Dynamics computing densitometer revealed that the total amount of Coomassie-blue-stained protein in Fig. 2 differed by less than 1%, enabling a quantitative comparison of the protein composition of normal and stimulated skeletal muscle by immunoblot analysis.

Immunoblot analysis of microsomal proteins in control and conditioned canine skeletal muscle

In order to identify changes in specific protein components, we carried out a detailed immunoblot analysis (Figs 3 and 4). Identical immunoblots of control and stimulated skel-

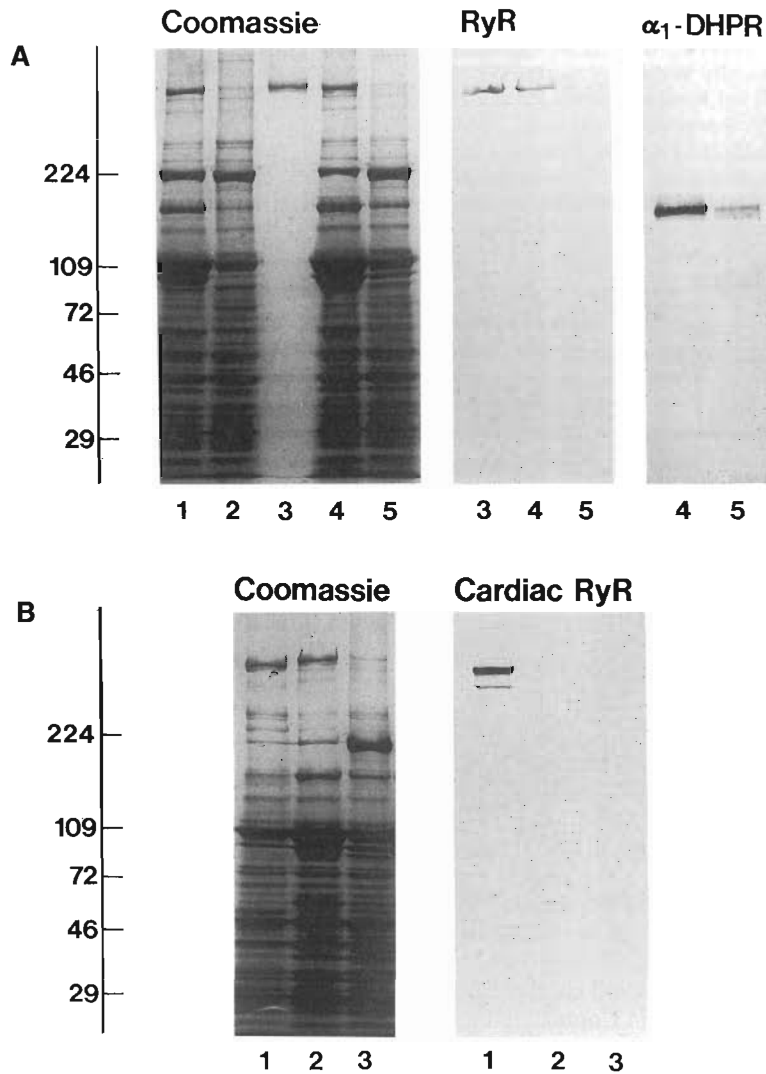

Fig. 3. Immunoblot analysis of RyR and DHPR in conditioned canine skeletal muscle. (A) A Coomassie-blue stained gel is shown and identical immunoblots of purified rabbit skeletal muscle RyR (lane 3), as well as microsomes from normal control (lanes 1 and 4) and conditioned (lanes 2 and 5) skeletal muscle from two different dogs. Immunoblots were stained with mAb XA7 against skeletal muscle RyR and mAb IIID5 against α_1 subunit of DHPR (α_1-DHPR). (B) A Coomassie-blue-stained gel is shown and an identical immunoblot of canine cardiac microsomes (lane 1), as well as microsomes from normal control (lane 2) and conditioned (lane 3) canine skeletal muscle. Immunoblot was stained with mAb RyR-1 against cardiac RyR. The same result was obtained using mAb RyR-3. Molecular mass standards are shown on the left.

etal muscle microsomes were labeled with specific antibodies against components of longitudinal SR and JSR, junctional transverse tubules and sarcolemma. To evaluate the usefulness of immunoblot analysis for the study of the expression of EC-coupling components we first investigated the relationship between protein content and quantitation of specific proteins. Various amounts of microsomes and isolated triads (0.05 – 200 µg protein) were separated by SDS/PAGE on 3 – 12% gradient gels. Antibody staining intensity from autoradiographs of immunoblots after incubation with ^{125}I-labeled secondary antibody revealed a linear relationship between the amount of protein and the relative labeling intensity of the specific antibody. These results show that immunoblot analysis is an excellent technique for investigating changes in protein expression in stimulated skeletal muscle. Fig. 3A illustrates the relative abundance of two EC-coupling components, the high-affinity RyR of the Ca^{2+}-release channel from JSR and the transverse tubular dihydropyridine-sensitive Ca^{2+} channel, in control and chronically stimulated canine skeletal muscle. Immunoblotting with mAb XA7 to the skeletal muscle RyR and mAb IIID5 to the skeletal muscle α_1-subunit of the DHPR demonstrates that the expression of both proteins is greatly suppressed in conditioned muscle compared to control. These results were highly reproducible in the six dogs studied. Estimation of the amount of RyR and DHPR remaining in the chronically stimulated muscle using ^{125}I-labelled secondary antibody revealed an approximate reduction of 87% and 83%, respectively (see Table 1). In densitometric scanning of autoradiographs, ^{125}I-secondary-antibody-labeled anti-sarcalumenin antibody was used as a control. Sarcalumenin of 160 kDa is not affected in conditioned muscle (Fig. 4) and can therefore be employed as a reliable control for loading equal amounts of microsomal protein from normal and conditioned muscle on the gel. Relative reduction of RyR in microsomes from stimulated muscle was confirmed by immunoblot analysis with sheep antisera

Table 1. Reduced expression of RyR and DHPR in chronically stimulated canine skeletal muscle. Microsomal membranes were prepared from untreated control and chronically stimulated canine skeletal muscle (latissimus dorsi) and analysed by autoradiography for the expression of the EC-coupling components RyR and DHPR. Immunoblots were labeled with mAb XA7 against RyR and mAb IIID5 against DHPR followed by incubation with ^{125}I-labeled secondary antibody. Reduction of RyR and DHPR expression in stimulated skeletal muscle was estimated by volume integration of autoradiographs with a Molecular Dynamics 300S computing densitometer using Image Quant V3.0 software.

Animal no.	Reduction in chronically stimulated canine skeletal muscle membranes	
	RyR (mAb XA7)	DHPR (mAb IIID5)
	%	
1	82	73
2	84	80
3	84	81
4	90	85
5	91	86
6	92	90

against skeletal muscle RyR (results not shown). Antibodies against other subunits of the DHPR did not cross-react with canine skeletal muscle proteins and could not, therefore, be investigated. The Coomassie-blue-stained gel of Fig. 3A compares the SDS/PAGE pattern of the two canine muscle fractions with purified RyR from rabbit skeletal muscle. The protein band of the high-molecular-mass RyR in normal skeletal muscle (Fig. 3A) corresponds to the protein band of purified RyR (Fig. 3A) which was isolated and characterized as described previously [3]. The reduction in Coomassie-blue-stained high-molecular-mass RyR protein band with chronic stimulation agrees with the findings from the immunoblot analysis (Fig. 3A). To investigate the possible appearance of a cardiac RyR isoform in conditioned muscle, membrane fractions were stained with mAb RyR-1 and RyR-3 against the cardiac RyR [24] (Fig. 3B). These mAb recognize only the cardiac form and exhibit no cross-reactivity with the skeletal muscle form of the receptor [24]. While canine cardiac microsomes were strongly labeled by mAb RyR-1 and RyR-3, no staining was observed in normal and stimulated skeletal muscle (Fig. 3B). Fig. 3B also illustrates that the cardiac RyR exhibits a slightly lower-molecular-mass than the skeletal muscle isoform as previously observed [24].

Chronic stimulation of a predominantly fast-twitch skeletal muscle enhances the expression of type-I (slow-twitch muscle) Ca^{2+}-ATPase and suppresses the expression of type-II (fast-twitch muscle) Ca^{2+}-ATPase [23] (Fig. 4) and can be used as a control for the successful transformation of the stimulated canine muscle. Successful fast-to-slow-twitch-fibre-type transition was also confirmed by indirect immunofluorescence microscopy carried out as described previously [23]. Labeling of identical immunoblots with antibodies against two JSR components of apparent molecular mass 90 kDa and 94 kDa revealed that the relative amount of both proteins is also greatly reduced (Fig. 4). While the Ca^{2+}-binding protein sarcalumenin of 160 kDa and a 53-kDa glycoprotein of SR appear to be unaffected by chronic stimulation (Fig. 4), staining with the cationic carbocyanine dye Stains-all demonstrates a suppressed expression of calsequestrin in stimulated muscle (Fig. 4). These findings agree with immunoblot staining of mAb VIIID1$_2$ to calsequestrin, which also demonstrates a reduced amount of calsequestrin in conditioned muscle (Fig. 4).

The fourth skeletal muscle membrane system investigated in normal and conditioned muscle was sarcolemma. mAb against sarcolemma marker Na^+/K^+-ATPase, dystrophin and DAG of 156 kDa revealed that these muscle surface membrane components were not significantly affected by chronic stimulation. All three sarcolemma proteins exhibited approximately equal staining intensity in normal control and conditioned canine skeletal muscle (Fig. 4).

DISCUSSION

This study investigates the effects of chronic stimulation on the function of skeletal muscle as assessed by alterations in membrane proteins from longitudinal SR and JSR, junctional transverse tubules and sarcolemma using immunoblot analysis. In contrast to the extensively investigated myofilament isozymes in transformed skeletal muscle [21], the effect of chronic stimulation on the expression of EC-coupling components and other muscle proteins involved in regulating calcium concentration has not been studied. Analysis of chronically stimulated rabbit fast-twitch muscle revealed an exchange of the fast-twitch with the slow-twitch Ca^{2+}-ATPase isoform mRNA [43]. These results agree with our findings of an enhanced expression of the slow Ca^{2+}-ATPase isoform in conditioned canine skeletal muscle, as obtained by immunoblot and immunofluorescence analysis [23]. The switch in Ca^{2+}-ATPase isoforms appears to be a reliable indication for a successful fast-to-slow-twitch transition in experimental skeletal muscle. These changes in SR are also illustrated by depressed Ca^{2+}-ATPase activity and Ca^{2+}-uptake of this membrane system in conditioned muscle [23, 44]. Furthermore, the suppressed expression of the SR Ca^{2+}-binding protein calsequestrin in conditioned muscle is comparable to its lower density in slow-twitch muscle and also illustrates the effect of chronic stimulation on skeletal muscle.

Here, we report for the first time a greatly reduced expression of important elements of EC coupling in chronically stimulated canine skeletal muscle. It is very interesting that two EC-coupling components, the RyR and the DHPR, which are localized on two different membrane systems, are both greatly reduced in conditioned muscle. The DHPR is thought to function as the voltage sensor that regulates the release of calcium ions via the RyR of the SR [2, 7, 8]. Our findings further supports the hypothesis that the RyR containing foot structures at the junction between the SR and the transverse tubules directly interact with the transverse tubule tetrad DHPR [6]. Appelt et al. [45] have reported that the length of junctional transverse tubules in the rat slow-twitch soleus is half that in fast-twitch extensor digitorum longus, whereas feet density/area of junctional transverse tubules is the same in the two types of fibres. Therefore, the amount of junctional transverse tubules and feet in slow-twitch fibres is approximately 50% of that in fast-twitch fibres. On the basis that the RyR and DHPR receptors are associated with these structures the expression of these proteins would be expected to be reduced, when compared to fast-twitch muscle, by about 50% in the slow-twitch vastus intermedius and the conditioned latissimus dorsi. The reduction in the conditioned muscle should exceed that of the vastus intermedius because the conditioned muscle is composed of 100% slow-twitch fibres [23].

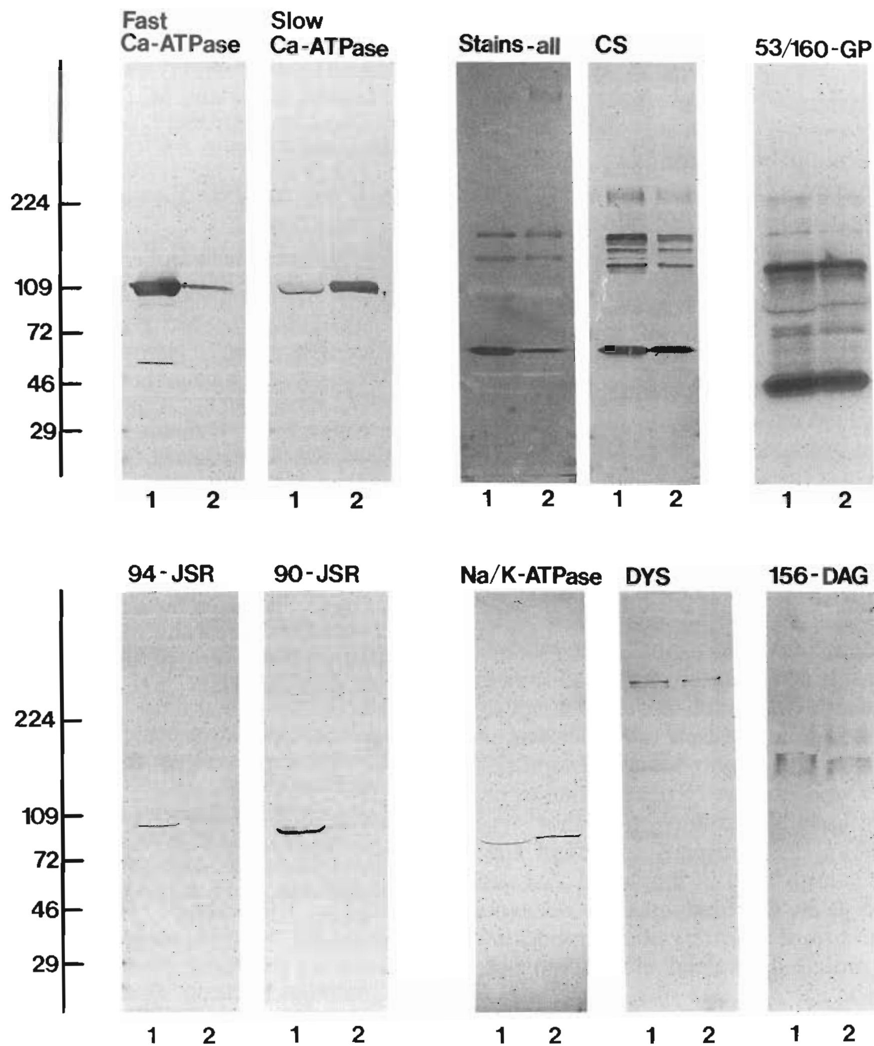

Fig. 4. Immunoblot analysis of microsomal proteins in control and conditioned canine skeletal muscle. A Stains-all-stained gel is shown and immunoblots (identical with the Coomassie-stained gel of Fig. 3A) of microsomes from normal control (lane 1) and conditioned (lane 2) canine skeletal muscle. Immunoblots were stained with mAb IIH11 against fast-twitch SR Ca^{2+}-ATPase, mAb IID8 against slow-twitch SR Ca^{2+}-ATPase, mAb VIIID1$_2$ against calsequestrin (CS), mAb XIIC4 against 160-kDa sarcalumenin and a 53-kDa glycoprotein (53/160-GP), guinea pig polyclonal antisera against JSR protein of 94 kDa (94-JSR), mAb VIIC12$_3$ against JSR protein of 90 kDa (90-JSR), mAb McB2 against Na^+/K^+-ATPase, mAb VIA4$_2$ against dystrophin (DYS) and mAb VIA4$_1$ against 156-kDa DAG (156-DAG). Molecular mass standards are shown on the left.

We have previously reported that the expression of the DHPR is 3–4-fold higher in fast-twitch rabbit muscle fibres than in slow-twitch muscle fibres, as expected from the distribution of junctional structures [25]. In the present study both the RyR and DHPR proteins were in lower concentration in the canine slow-twitch fiber, vastus intermedius (Fig. 1), and in the conditioned muscle (Fig. 3), as would occur if the structure of junctional transverse tubules and feet in the dog were similar to that in rat. Most likely transformation from fast-twitch to slow-twitch muscle causes a significant decrease of transverse tubule and terminal cisternae surface area, but this question can better be investigated by analysis with electron microscopy. The levels of expression of the RyR and DHPR in the vastus intermedius and conditioned latissimus dorsi support the hypothesis that chronic stimulation transforms fast-twitch to slow-twitch muscle fibres. Imagawa et al. [24] showed that the cardiac isoform of the RyR is absent in fast-twitch (type II) and slow-twitch (type I) skeletal muscle, which is not the case for other cardiac isoforms, i.e. the slow-twitch muscle Ca^{2+}-ATPase is immunologically cross-reactive with the cardiac isoform [29]. Immunoblot labeling with mAb against the cardiac RyR revealed the absence of the cardiac isoform in our conditioned muscle, indicating that the transformed muscle exhibits slow muscle characteristics, but not cardiac-like isoforms with respect to RyR. We could not study the presence of cardiac-specific subunits of the DHPR in the transformed muscle samples due to the lack of antibodies to the cardiac receptor. The reduced expression in conditioned muscle of the JSR proteins of apparent 90 kDa and 94 kDa further indicates that proteins possibly involved in EC-coupling are affected in the fast-to-slow-twitch muscle transition. Other reports have described JSR proteins of similar molecular mass which are possibly in close proximity to the RyR Ca^{2+}-release channel at the junctional face membrane of SR [46–48]. These proteins could have regulatory functions in EC coupling or Ca^{2+} release.

In contrast, the Ca^{2+}-binding protein sarcalumenin and a 53-kDa glycoprotein of SR are not affected by chronic

stimulation of skeletal muscle. Both proteins are possibly involved in the transfer of Ca^{2+} from the Ca^{2+}-transport sites in the non-junctional regions of the SR to the sequestering sites localized in the terminal cisternae [31]. The relative amount of these two SR components appears to be independent of muscle fibre type and is not affected in conditioned skeletal muscle. Furthermore, marker proteins of the sarcolemma are not affected in the fast-to-slow-twitch muscle transition. All three sarcolemma components investigated in this study, Na^{+}/K^{+}-ATPase, dystrophin and its associated glycoprotein of apparent 156 kDa, are equally distributed between the different muscle fibre types as revealed by immunofluorescence microscopy [30] and immunoblot analysis (Fig. 4). This might explain why the expression of these sarcolemma components is not affected in conditioned muscle. We did not investigate changes in mitochondrial proteins, but most likely fibre type changes also entail changes in the abundance of mitochondria. A prerequisite for a detailed analysis of mitochondrial and metabolic enzymes in conditioned muscle is the availability of highly specific and immunological cross-reactive antibodies to these components which are not accessible to us at this time.

Our analysis of EC-coupling components in fast-to-slow-twitch muscle transformation is relevant for the possible medical application of transformed skeletal muscle. Previously, experimentally constructed skeletal muscle ventricles demonstrated the potential to directly support the circulation [22]. Skeletal muscle ventricles were prepared from preconditioned canine latissimus dorsi muscle and connected in short-term experiments to the animal's systemic arterial circulation. Conditioned skeletal muscle can possibly be employed as a supporting muscle pump to assist the function of a weakened heart muscle. Of basic importance for the medical application of conditioned skeletal muscle as skeletal muscle ventricles is a successful and reproducible fast-to-slow-twitch muscle transformation under standardized conditions. Our extensive immunoblot analysis of important EC-coupling components and other muscle membrane proteins addresses this question and implies that a successful transformation of the latissimus dorsi is accomplished using chronic stimulation at 2 Hz via the thoracodorsal nerve as described [22].

In conclusion, chronic stimulation of a predominantly fast-twitch canine skeletal muscle affects, besides the fast-twitch Ca^{2+}-ATPase of sarcoplasmic reticulum, important components of EC coupling. Comparison of the conditioned muscle with the predominantly slow-twitch muscle, the vastus intermedius, indicates that the EC phenotype, as judged by the expression of the RyR and DHPR, is comparable. Possibly the changes in the expression of EC-coupling components account for different contractile properties of slow-twitch fibres compared to fast-twitch fibres. Therefore fast-twitch and slow-twitch muscles are not only characterized by differences in contractile and regulatory myofibrillar proteins, but also differ clearly in the abundance of components involved in EC-coupling. It will be interesting to learn the physiological significance of these changes in the EC-coupling apparatus.

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