ELSEVIER

Biochimica et Biophysica Acta 1466 (2000) 151–168

BIOCHIMICA ET BIOPHYSICA ACTA

BBA

www.elsevier.com/locate/bba

Comparative analysis of the isoform expression pattern of Ca^{2+}-regulatory membrane proteins in fast-twitch, slow-twitch, cardiac, neonatal and chronic low-frequency stimulated muscle fibers

Gabriele R. Froemming [a], Brendan E. Murray [a], Shona Harmon [a], Dirk Pette [b], Kay Ohlendieck [a,*]

[a] *Department of Pharmacology, Conway Institute of Biomolecular and Biomedical Research, University College Dublin, Belfield, Dublin 4, Ireland*

[b] *Faculty of Biology, University of Konstanz, Postfach 5560, D-78434 Konstanz, Germany*

Received 16 December 1999; received in revised form 6 March 2000; accepted 14 March 2000

Abstract

Although all muscle cells generate contractile forces by means of organized filament systems, isoform expression patterns of contractile and regulatory proteins in heart are not identical compared to developing, conditioned or mature skeletal muscles. In order to determine biochemical parameters that may reflect functional variations in the Ca^{2+}-regulatory membrane systems of different muscle types, we performed a comparative immunoblot analysis of key membrane proteins involved in ion homeostasis. Cardiac isoforms of the α_1-dihydropyridine receptor, Ca^{2+}-ATPase and calsequestrin are also present in skeletal muscle and are up-regulated in chronic low-frequency stimulated fast muscle. In contrast, the cardiac RyR2 isoform of the Ca^{2+}-release channel was not found in slow muscle but was detectable in neonatal skeletal muscle. Up-regulation of RyR2 in conditioned muscle was probably due to degeneration–regeneration processes. Fiber type-specific differences were also detected in the abundance of auxiliary subunits of the dihydropyridine receptor, the ryanodine receptor and the Ca^{2+}-ATPase, as well as triad markers and various Ca^{2+}-binding and ion-regulatory proteins. Hence, the variation in innervation of different types of muscle appears to have a profound influence on the levels and pattern of isoform expression of Ca^{2+}-regulatory membrane proteins reflecting differences in the regulation of Ca^{2+}-homeostasis. However, independent of the muscle cell type, key Ca^{2+}-regulatory proteins exist as oligomeric complexes under native conditions.

Keywords: Ryanodine receptor; Dihydropyridine receptor; Calsequestrin; Excitation–contraction coupling; Chronic low-frequency stimulation

1. Introduction

There exists a great diversity not only in the physiological regulation of excitation, contraction, relaxation, energy metabolism and ion homeostasis between the main types of muscle, i.e. skeletal muscle, cardiac muscle, smooth muscle and myoepithelial cells, but also between sub-types of skeletal muscle fibers. Separate groups of skeletal muscles differ in their cellular heterogeneity in fiber types and also in distinct variations on the molecular level of individual muscle cells [1]. Differences in levels and expres-

* Corresponding author. Fax: +353-1-269-2749; E-mail: kay.ohlendieck@ucd.ie

0005-2736/00/$ – see front matter
PII: S0005-2736(00)00195-4

sion of muscle-specific isoforms reflect distinct biological adaptations to specific functional demands [2]. Based on immunohistochemical evaluations [3] and histochemical assays exploiting the pH stability/lability of myofibrillar ATPase [4], several different skeletal muscle fiber types can be differentiated. Besides qualitative histochemistry and immunohistochemistry, the more recent application of quantitative micro-methods including microphotometry, microbiochemical and micromolecular biological assays, have decisively increased our understanding of the enormous molecular complexity of skeletal muscle fibers [5]. The myosin heavy chain content of different skeletal muscle cells correlates reasonably well with myofibrillar ATPase-based fiber typing [1,2]. Each histochemically identifiable skeletal muscle fiber seems to exhibit a highly specific expression profile of myosin heavy chains. This unique cellular distribution of a muscle-specific protein has been the basis of a series of studies into molecular changes of the contractile apparatus during fiber type transitions induced by the physiological model system of chronic low-frequency stimulation of fast muscles [6]. A gradual replacement of myosin heavy chains follows the sequence of MHCIIb to MHCIId(x) to MHCIIa to MHCI during fast-to-slow transformation [7]. In contrast to the stepwise isoform exchange of myofibrilliar proteins, relatively little is known about the expression of cardiac isoforms of Ca^{2+}-channels in chronically conditioned skeletal muscle fibers.

Skeletal muscle Ca^{2+}-channels play an important role as voltage sensors or Ca^{2+}-release channels in excitation–contraction coupling [8,9]. We could show previously that the expression of skeletal muscle isoforms of the transverse-tubular voltage sensor, the α_{1S}-subunit of the dihydropyridine receptor, and the Ca^{2+}-release channel of the junctional sarcoplasmic reticulum, the ryanodine receptor RyR1 isoform, are drastically reduced following chronic low-frequency stimulation [10–12]. Down-regulation of fast skeletal muscle isoforms might be partially compensated by an increase in slow/cardiac isoforms, since electro-stimulation triggers the up-regulation of mRNA encoding for the cardiac isoform of the dihydropyridine receptor [13]. In contrast to earlier assumptions, recent studies have also shown that both diaphragm and soleus muscle express mRNA for the cardiac isoform of this Ca^{2+}-channel at a significant level [14]. To complement these investigations on the protein level, in this study we have performed an analysis of the expression patterns of the cardiac isoforms of the voltage sensor and the Ca^{2+}-release channel, i.e. the α_{1C}-subunit of the dihydropyridine receptor and the RyR2 isoform of the ryanodine receptor. These comparative investigations may be important for our general understanding of the molecular events occurring during skeletal muscle adaptations to changed innervation patterns. In addition, they might point out differences on the level of protein expression that reflect variations in the physiological fine-regulation of Ca^{2+}-homeostasis between differing muscle types.

Excitation–contraction coupling, a unique signal transduction event in which depolarization of the sarcolemma leads to Ca^{2+}-release from the sarcoplasmic reticulum, clearly differs between skeletal and cardiac muscle, slow-twitch and fast-twitch fibers, neonatal and adult muscle cells, as well as between unconditioned and chronic low-frequency stimulated fast muscle. In adult skeletal muscle, excitation–contraction coupling is thought to be mediated by direct physical interactions between the voltage-sensing α_1-dihydropyridine receptor and the sarcoplasmic reticulum Ca^{2+}-release channel complex [15]. In contrast, in cardiac muscle fibers Ca^{2+}-ions enter initially the cytosol via activated dihydropyridine receptors, which in turn triggers a Ca^{2+}-induced Ca^{2+}-release mechanism involving the RyR2 isoform of the ryanodine receptor [16]. On the molecular level, the differential expression of sub-types of dihydropyridine receptors reflects the two different modes of excitation–contraction coupling, the cardiac or the skeletal muscle signal transduction mechanism [17]. In the early stages of skeletal muscle development, a physiological dependency of excitation–contraction coupling from a cardiac-like external Ca^{2+}-flux is present, but this type of signal transduction mechanism declines in its importance during later stages of postnatal skeletal muscle development [18]. Developing muscles express a variety of cardiac/slow isoforms abundantly, e.g. the Ca^{2+}-ATPase isoform SERCA2 of the sarcoplasmic reticulum, the Ca^{2+}-binding protein sCSQ-isoform of the terminal cisternae and the dihydropyridine receptor α_{1C}-subunit

isoform of the junctional transverse tubules [18–21]. During postnatal myogenesis these Ca^{2+}-regulatory isoforms are down-regulated and their respective fast skeletal muscle isoforms increase in relative density [21]. In the first two weeks after birth, excitation–contraction coupling is therefore thought to be more cardiac-like, but following maturation of the triad junctions, skeletal muscle fibers exhibit physical interactions between the RyR1 isoform and the α_{1S}-subunit of the dihydropyridine receptor [22].

Thus, in order to directly compare the expression patterns of key Ca^{2+}-regulatory muscle proteins and associated membrane components, we have performed an immunoblot analysis of microsomal proteins derived from predominantly fast-twitch muscle, predominantly slow-twitch muscle, heart muscle and neonatal skeletal muscle, as well as chronic low-frequency stimulated fast-twitch muscle fibers. To unequivocally identify the various isoforms of muscle proteins, monoclonal and sequence-specific polyclonal antibodies were employed for highly specific immunodecoration. Our analysis included the α_{1S}-, α_{1C}-, α_2- and β-subunits of the transverse-tubular dihydropyridine receptor [23], the RyR1 and RyR2 isoforms of the sarcoplasmic reticulum Ca^{2+}-release channel [24] and their regulatory FKBP12-subunit [25], Ca^{2+}-binding proteins such as fast and slow calsequestrin of the terminal cisternae [26], sarcalumenin of the longitudinal tubules [27] and calreticulin [28], the junctional triad markers of 90 [29] and 94 kDa (triadin) [30], as well as the slow and fast Ca^{2+}-ATPase [31], phospholamban [32], the Na^+/Ca^{2+}-exchanger [33] and the Na^+/K^+-ATPase [34]. Immunodecoration of the fast and slow isoforms of the myosin heavy chain [35] was used for the internal standardization of immunoblots.

2. Materials and methods

2.1. Materials

Peroxidase-conjugated secondary antibodies and protease inhibitors were purchased from Boehringer-Mannheim (Lewis, UK). The chemical cross-linker dithiobis-succinimidyl-propionate, as well as chemiluminescence substrates were obtained from Pierce and Warriner (Chester, UK). Immobilon NC nitrocellulose membranes were from Millipore (Bedford, MA, USA). All other chemicals were of analytical grade and purchased from Sigma (Dorset, UK).

2.2. Antibodies

The specificity of antibodies used in this study to differentiate between varying isoform expression patterns is summarized in Table 1. For the production of polyclonal antisera against the β-subunit of the dihydropyridine receptor [36], a peptide representing the last 15 residues of the carboxy-terminus of this muscle protein was coupled to keyhole limpet hemocyanin carrier protein by Research Genetics (Huntington, AL, USA). Sequence-specific antibodies were raised by four monthly injections, with the use of a standard immunization protocol [37]. Monoclonal antibodies IIID5 against the α_{1S}-subunit of the dihydropyridine receptor [12], $VIIID1_2$ against fast calsequestrin [38] and IIG12 against triadin [30] were a generous gift from Dr. Kevin P. Campbell (Howard Hughes Medical Institute, University of Iowa, Iowa City, IA, USA). Polyclonal antibodies against slow calsequestrin [12], calreticulin [12] and FKBP12 [21], as well as monoclonal antibodies 20A, IIH11, IID8, XIIC4, VF1c, C2C12, 2D12 and C3-33 against the α_2-subunit of the dihydropyridine receptor [39], the fast SERCA1 isoform of the Ca^{2+}-ATPase [10], the slow SERCA2 isoform of the Ca^{2+}-ATPase [10], sarcalumenin/53 kDa glycoprotein [12], the 90 kDa junctional face membrane protein [29], the Na^+/Ca^{2+}-exchanger [40], phospholamban [41] and the cardiac RyR2 isoform of the Ca^{2+}-release channel [39], respectively, were purchased from Affinity Bioreagents (Golden, CO, USA). Monoclonal antibody C464.6 against the α_1-Na^+/K^+-ATPase [12] and polyclonal antibodies to the RyR1 [21] and RyR3 isoforms of the Ca^{2+}-release channel were from Upstate Biotechnology (Lake Placid, NY, USA). Monoclonal antibodies MY-33 and NOQ7.5-4D against the fast and slow myosin heavy chain [35] were obtained from Sigma (Dorset, UK) and a polyclonal antibody against the cardiac α_{1C}-subunit of the dihydropyridine receptor [42] was from TCS Biologicals (Buckingham, UK).

2.3. Chronic low-frequency stimulation

Conditioning of the left hind limb of adult male New Zealand white rabbits was performed in the Animal Facility of the University of Konstanz using chronic telestimulation through the peroneal nerve [43]. For the analysis of effects of electro-stimulation on the isoform expression pattern of key Ca^{2+}-regulatory membrane proteins, the tibialis anterior muscle was continuously stimulated at 10 Hz for 4 and 30 days. In addition, in order to determine whether discontinuation of chronic electro-stimulation results in a reversal of conditioning-induced effects, a recovery study was carried out. Following 14 days of telestimulation, conditioning of the tibialis anterior was terminated and the muscle left unstimulated for 30 days prior to analysis. Animals were killed by cervical dislocation and the tibialis anterior was excised, cut into longitudinal sections and quick-frozen in liquid nitrogen. Samples were stored at −70°C prior to further usage.

2.4. Preparation of microsomal membranes

For routine membrane preparations, New Zealand white rabbits were obtained from the Biomedical Facility of the University College Dublin, Ireland. All preparative steps were performed at 0–4°C in the presence of a protease inhibitor cocktail [36]. The microsomal fraction was isolated by standard subcellular fractionation methodology from various rabbit muscle tissues [21]: mature soleus, tibialis anterior and extensor digitorum longus muscles, adult heart muscle, neonatal skeletal muscle (5 days after birth), as well as 4 and 30 days chronic low-frequency stimulated tibialis anterior muscle. The immunoblot analyses shown in this study illustrate representative results from experimental data received from membrane preparations of different tissue types from four different animals. For recovery studies, microsomes were prepared from 14 days electro-stimulated tibialis anterior with or without a 30 days unstimulated recovery period. Membrane samples were immediately used for gel electrophoresis or chemical crosslinking analysis or otherwise quick-frozen in liquid nitrogen and then stored at −70°C.

2.5. Chemical crosslinking

In order to determine potential differences in pro-

Table 1
Isoform-specific antibodies to muscle proteins

Muscle protein	Isoform specificity	Antibody	Reference
Myosin heavy chain	fast MHC_f	mAb MY-33	[35]
Myosin heavy chain	slow MHC_s	mAb NOQ7.5-4D	[35]
Dihydropyridine receptor	α_{1S}-DHPR	mAb IIID5	[12]
Dihydropyridine receptor	α_{1C}-DHPR	polyclonal	[42]
Dihydropyridine receptor	α_2-DHPR	mAb 20A	[39]
Dihydropyridine receptor	β-DHPR	polyclonal	[36]
Ca^{2+}-release channel	RyR1	polyclonal	[21]
Ca^{2+}-release channel	RyR2	mAb C3-33	[39]
Ryanodine receptor subunit	FKBP12	polyclonal	[21]
Triadin	fast TRI	mAb IIG12	[30]
Sarcalumenin	fast SAR	mAb XIIC4	[12]
SR glycoprotein	53 kDa SR GP	mAb XIIC4	[12]
Junctional face protein	90 kDa JFP	mAb VF1c	[29]
Ca^{2+}-ATPase	fast SERCA1	mAb IIH11	[10]
Ca^{2+}-ATPase	slow SERCA2	mAb IID8	[10]
Phospholamban	PLN	mAb 2D12	[41]
Calsequestrin	fast CSQ	mAb $VIIID1_2$	[38]
Calsequestrin	slow CSQ	polyclonal	[12]
Calreticulin	CAL	polyclonal	[12]
Na^+/Ca^{2+}-exchanger	Na^+/Ca^{2+}-exchanger	mAb C2C12	[40]
Na^+/K^+-ATPase	α_1- Na^+/K^+-ATPase	mAb C464.6	[12]

tein–protein interactions between cardiac and skeletal muscle isoforms in fast muscle, heart, neonatal skeletal muscle and chronic low-frequency stimulated fast muscle, chemical crossslinking experiments were performed by an established protocol [36]. Since this analysis could only be performed with relatively abundant Ca^{2+}-regulatory membrane proteins for a reliable immunodetection, oligomerization of the RyR1 isoform of the Ca^{2+}-release channel and the SERCA1 isoform of the Ca^{2+}-ATPase was compared with that of their slow counterparts, the RyR2 and the SERCA2 isoforms. Microsomal fractions were treated at room temperature in 50 mM HEPES buffer at pH 8.0 for 30 min with 100 μg dithiobis-succinimidyl-propionate per mg protein. A crosslinking-induced decrease in relative electrophoretic mobility was determined in the case of skeletal muscle isoforms for tibialis anterior muscle, neonatal muscle and 4 days chronic low-frequency stimulated tibialis anterior, and in the case of cardiac isoforms for heart muscle, neonatal muscle and 4 days chronic low-frequency stimulated tibialis anterior. The crosslinking reaction was terminated using 50 μl of 1 M ammonium acetate per ml of reaction medium. Subsequently, samples were solubilized with sodium dodecyl sulfate-containing sample buffer under non-reducing conditions [44] and proteins were separated by gel electrophoresis.

2.6. Gel electrophoresis and immunoblotting

To determine the isoform expression pattern of muscle proteins, electrophoretic separation of microsomes was carried out under reducing conditions by standard sodium dodecyl sulfate–polyacrylamide gel electrophoresis [44] using 5, 7 or 8% (w/v) gels with 20 μg protein per lane. For the analysis of crosslinking-induced changes in the relative molecular mass of protein complexes, electrophoresis was performed under non-reducing conditions. Employing a Bio-Rad Mini-Protean II system (Bio-Rad Laboratories, Hemel Hempstead, UK), gels were run for 280 or 360 Vh at constant voltage, depending on the relative molecular mass of the proteins to be analyzed. Following electrophoretic separation, muscle proteins were transferred onto Immobilion NC nitrocellulose membranes according to Towbin et al. [45] using a Bio-Rad Mini-Protean blotting system (Bio-Rad Laboratories, Hemel Hempstead, UK). Blocking and incubation of nitrocellulose sheets with primary and peroxidase-conjugated secondary antibodies was carried out as previously described in detail [21]. The enhanced chemiluminescence detection method was used to visualize immunodecorated protein bands [46].

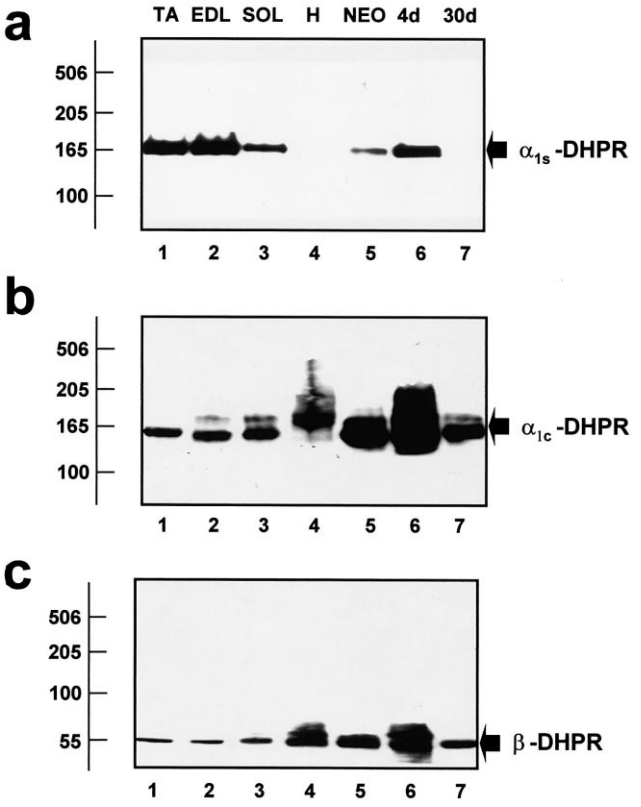
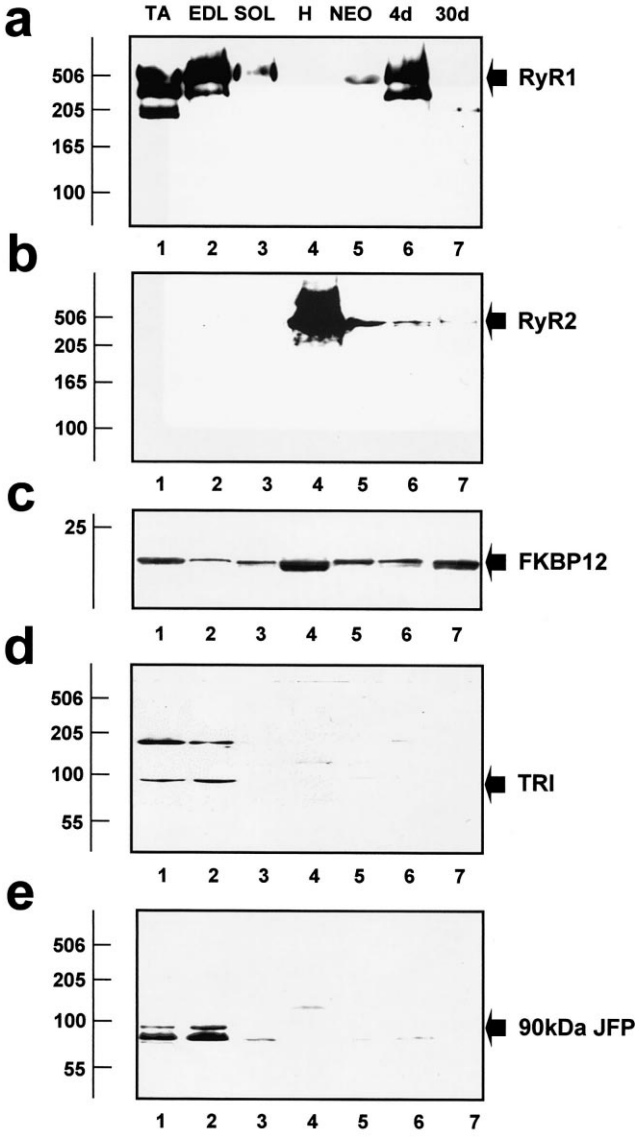


Fig. 1. Expression pattern of the dihydropyridine receptor in muscle tissues. Shown are immunoblots stained with antibodies to the α_{1S}-subunit (a), the α_{1C}-subunit (b), and the β-subunit (c) of the dihydropyridine receptor (DHPR) complex. See Section 2 and Table 1 for a detailed description of antibodies used. Lanes 1–7 represent the microsomal fraction isolated from mature tibialis anterior (TA), mature extensor digitorum longus (EDL), mature soleus (SOL), adult heart (H), neonatal skeletal muscle (NEO), as well as 4 days (4d) and 30 days (30d) chronic low-frequency stimulated tibialis anterior muscle, respectively. Proteins were separated on 5% (w/v) gels for the analysis of α_{1S}-DHPR and α_{1C}-DHPR and on 7% (w/v) gels for the analysis of β-DHPR, and then transferred onto nitrocellulose. Molecular weight markers are indicated on the left and individual proteins are marked by arrows on the right.

3. Results

To determine differences in the amount and isoform expression pattern of Ca^{2+}-regulatory muscle membrane proteins, microsomal preparations isolated from mature tibialis anterior, extensor digitorum longus and soleus muscles, adult heart muscle, neonatal skeletal muscle and chronic electro-stimulated tibialis anterior muscle were compared by immunoblot analysis. Muscle isoforms involved in the regulation and stabilization of the excitation–contraction–relaxation cycle of muscle fibers, i.e. the voltage-sensing dihydropyridine receptor (Fig. 1), the ryanodine receptor Ca^{2+}-release channel and its FKBP12 subunit, triadin and the 90 kDa junctional face membrane protein (Fig. 2), the Ca^{2+}-binding proteins calsequestrin, calreticulin and sarcalumenin (Fig. 3) and the ion-regulatory components Ca^{2+}-ATPase, phospholamban, Na^+/Ca^{2+}-exchanger and the Na^+/K^+-ATPase (Fig. 4) were studied. The well-established distribution pattern of the fast and slow isoforms of the myosin heavy chain (Fig. 5) are shown for comparative purposes. Recovery studies with chronically electro-stimulated muscles (Fig. 6) and chemical crosslinking analyses (Fig. 7) are presented to illustrate the plasticity of muscle fibers and the importance of protein–protein interactions within Ca^{2+}-regulatory membrane complexes, respectively.



3.1. Expression pattern of the dihydropyridine receptor in muscle tissues

Since functional differences exist between the major cardiac and skeletal muscle α_1-subunit of the dihydropyridine receptor, i.e. the α_{1S}- and the α_{1C}-isoforms, their expression pattern in various muscle types was investigated. Fig. 1 shows the relative distribution of the α_{1S}-, α_{1C}- and β-subunit of this transverse-tubular receptor complex. The α_{1S}-subunit of apparent 175 kDa was clearly found in all unstimulated skeletal muscle fibers. Its expression was highest in tibialis anterior and extensor digitorum longus muscles, of lower abundance in slow-twitching soleus

←

Fig. 2. Expression pattern of the ryanodine receptor and other triad markers in muscle tissues. Shown are immunoblots stained with antibodies to the RyR1 (a) and RyR2 (b) isoforms of the sarcoplasmic reticulum Ca^{2+}-release channel, the immunophilin FK506-binding protein of apparent 12 kDa (FKBP12) (c), triadin (TRI) (d) and the 90 kDa junctional face membrane protein (90 kDa JFP) (e). See Section 2 and Table 1 for a detailed description of antibodies used. Lanes 1–7 represent the microsomal fraction isolated from mature tibialis anterior (TA), mature extensor digitorum longus (EDL), mature soleus (SOL), adult heart (H), neonatal skeletal muscle (NEO), as well as 4 days (4d) and 30 days (30d) chronic low-frequency stimulated tibialis anterior muscle, respectively. Proteins were separated on 5% (w/v) gels for the analysis of RyR1 and RyR2, on 7% (w/v) gels for the analysis of triadin and the 90 kDa junctional face membrane protein and on 8% (w/v) gels for the analysis of FKBP12, and then transferred onto nitrocellulose. Molecular weight markers are indicated on the left and individual proteins are marked by arrows on the right.

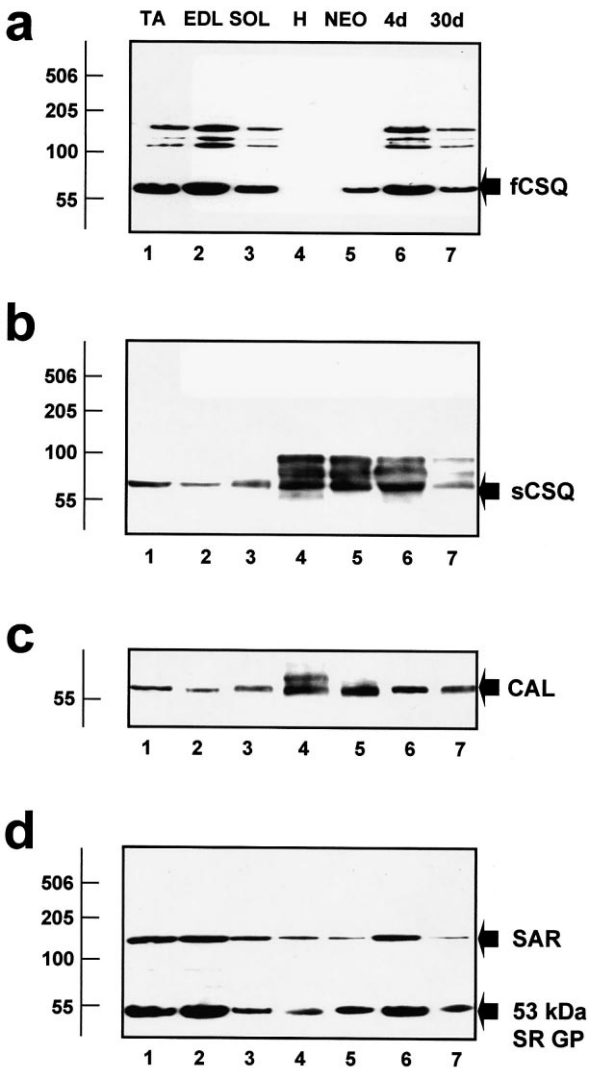


Fig. 3. Expression pattern of Ca^{2+}-reservoir proteins in muscle tissues. Shown are immunoblots stained with antibodies to the fast isoform of calsequestrin (fCSQ) (a), the slow/cardiac isoform of calsequestrin (sCSQ) (b), calreticulin (CAL) (c) and sarculumenin (SAR) and its immunologically related 53 kDa sarcoplasmic reticulum glycoprotein (53 kDa SR GP) (d). See Section 2 and Table 1 for a detailed description of antibodies used. Lanes 1–7 represent the microsomal fraction isolated from mature tibialis anterior (TA), mature extensor digitorum longus (EDL), mature soleus (SOL), adult heart (H), neonatal skeletal muscle (NEO), as well as 4 days (4d) and 30 days (30d) chronic low-frequency stimulated tibialis anterior muscle, respectively. Proteins were separated on 7% (w/v) gels and then transferred onto nitrocellulose. Molecular weight markers are indicated on the left and individual proteins are marked by arrows on the right.
←

and neonatal skeletal muscle and completely absent in the heart (Fig. 1a). In contrast, the α_{1C}-subunit of the dihydropyridine receptor was present at high concentration in heart and neonatal skeletal muscle, at intermediate levels in slow-twitch skeletal muscle fibers, and at its lowest level in predominantly fast-twitch fibers (Fig. 1b). Between the various muscle types investigated, no major difference in the expression was found for the α_2-subunit of apparent 143 kDa (not shown). In contrast, the regulatory β-subunit of approximately 54 kDa showed elevated levels in neonatal skeletal muscle and heart as compared to mature skeletal muscle fibers (Fig. 1c).

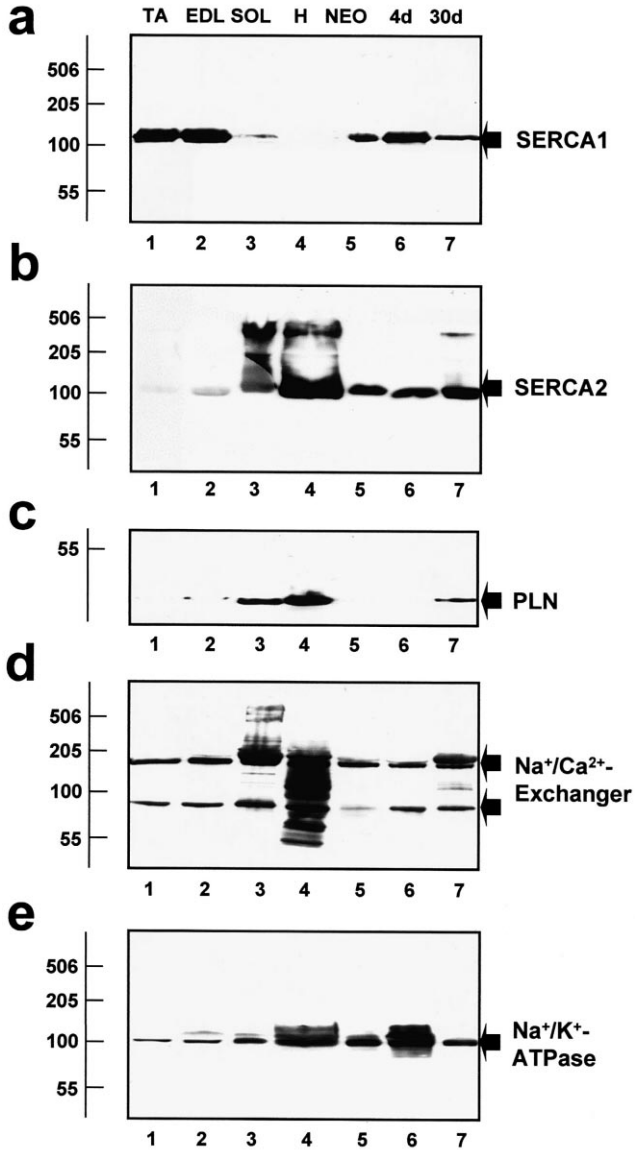
Chronic low-frequency stimulation had a dramatic effect on the level and expression pattern of the principal subunit of the dihydropyridine receptor. In agreement with earlier studies on stimulation-induced changes in Ca^{2+}-regulatory membrane complexes [10–13], the relative amount of the α_{1S}-subunit declined after 4 days of conditioning (Fig. 1a). However, for the first time we can show here that this decrease in the skeletal muscle-type voltage-sensor is compensated by an up-regulation of the cardiac-type α_{1C}-subunit on the protein level (Fig. 1b). Interestingly, the abundance of the α_{1C}-subunit of the dihydropyridine receptor is higher following 4 days of stimulation as compared to 30 days of conditioning (Fig. 1b). Immunoblotting of the β-subunit revealed a very similarly increased expression level of this protein during the fast-to-slow transition process as was observed for the α_{1C}-subunit (Fig. 1d). The α_2-subunit of the dihydropyridine receptor showed only a slight decrease in its expression level following chronic electro-stimulation (not shown), whereby this effect was much less dramatic than that described above for the α_{1S}-subunit of this Ca^{2+}-channel (Fig. 1a).

3.2. Expression pattern of the ryanodine receptor and other triad markers in muscle tissues

Another muscle Ca^{2+}-channel involved in excitation–contraction coupling is the ryanodine receptor Ca^{2+}-release channel of apparent 565 kDa. Since three main isoforms of the ryanodine receptor exist, we investigated the expression of the major skeletal muscle form and the cardiac-specific protein. In anal-

ogy to the findings for the α_{1S}-subunit of the dihydropyridine receptor (Fig. 1a), the expression of the RyR1 isoform of the sarcoplasmic reticulum Ca^{2+}-release channel was highest in tibialis anterior and extensor digitorum longus muscles, of much lower abundance in soleus and neonatal skeletal muscle, and completely absent in the heart (Fig. 2a). Besides the major RyR1 band of apparent 565 kDa, lower molecular mass bands of approximately 200 to 400 kDa were also immuno-labeled. These minor RyR bands probably represent degradation products and/or smaller isoforms of this triad receptor. Immunodecoration of the RyR2 isoform revealed a high amount of this Ca^{2+}-channel in heart microsomes, low levels in neonatal skeletal muscle and none in mature skeletal muscle (Fig. 2b). Labeling of identical nitrocellulose sheets, as used for Fig. 2a, with a polyclonal antibody to the predominant brain RyR3 isoform, did not result in any reliable immuno-staining above background (results not shown). We could therefore not investigate the expression pattern of this ryanodine receptor in different types of muscle fibers.

On the other hand, antibodies to an auxiliary component of the Ca^{2+}-release channel, the FKBP12 component of apparent 12 kDa, showed expression of this sarcoplasmic reticulum protein in all muscle tissue tested (Fig. 2c). Its highest abundance was in cardiac muscle fibers (Fig. 2c). FKBP12 exists as two isoforms, FKBP12 and FKBP12.6 [47], which bind to the RyR1 and RyR2 isoforms, respectively. However, the polyclonal antibody used does not distinguish between these two different protein species, since both isoforms are very similar in their primary structure. Two markers of the triad junction, triadin of 94 kDa and a novel component of slightly lower relative molecular mass, the 90 kDa junctional face membrane protein, were both detected in predominantly fast-twitching muscles (Fig. 2d,e). Besides the 94 kDa band, a second band of apparent 200 kDa was recognized by monoclonal antibody IIG12 against triadin, possibly representing a dimer (Fig. 2d). Triadin exists under native conditions as a high-molecular-mass cluster of approximately 3000 kDa [30] and tightly associated dimers might exist



←

Fig. 4. Expression pattern of ion-regulatory membrane proteins in muscle tissues. Shown are immunoblots stained with antibodies to the fast SERCA1 isoform of the sarcoplasmic reticulum Ca^{2+}-ATPase (a), the slow/cardiac SERCA2 isoform of the sarcoplasmic reticulum Ca^{2+}-ATPase (b), the regulatory SERCA2 subunit phospholamban (PLN) (c), the Na^+/Ca^{2+}-exchanger (d) and the Na^+/K^+-ATPase (e). See Section 2 and Table 1 for a detailed description of antibodies used. Lanes 1–7 represent the microsomal fraction isolated from mature tibialis anterior (TA), mature extensor digitorum longus (EDL), mature soleus (SOL), adult heart (H), neonatal skeletal muscle (NEO), as well as 4 days (4d) and 30 days (30d) chronic low-frequency stimulated tibialis anterior muscle, respectively. Proteins were separated on 7% (w/v) (a, b, d, e) or 8% (w/v) gels (c), and then transferred onto nitrocellulose. Molecular weight markers are indicated on the left and individual proteins are marked by arrows on the right.

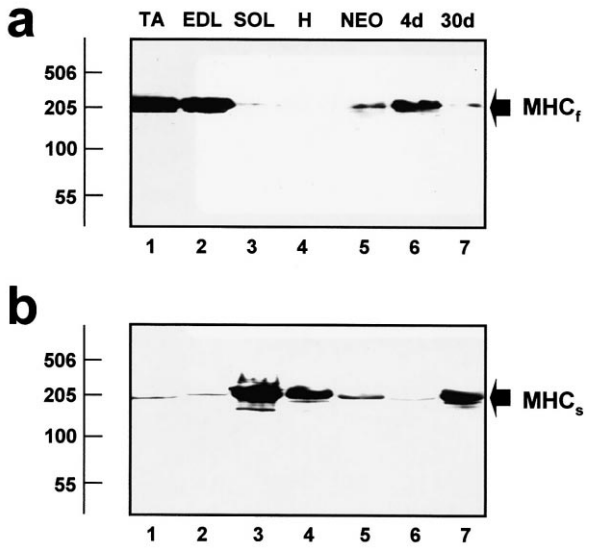


Fig. 5. Expression pattern of fast and slow myosin heavy chains in muscle tissues. Shown are immunoblots stained with antibodies to the fast (MHC_f) (a) and slow (MHC_s) (b) isoform of the myosin heavy chain (MHC). See Section 2 and Table 1 for a detailed description of antibodies used. Lanes 1–7 represent the microsomal fraction isolated from mature tibialis anterior (TA), mature extensor digitorum longus (EDL), mature soleus (SOL), adult heart (H), neonatal skeletal muscle (NEO), as well as 4 days (4d) and 30 days (30d) chronic low-frequency stimulated tibialis anterior muscle, respectively. Proteins were separated on 7% (w/v) gels, and then transferred onto nitrocellulose. Molecular weight markers are indicated on the left and individual proteins are marked by arrows on the right.

even under chemically reducing conditions. Both sarcoplasmic reticulum proteins were not present in neonatal skeletal muscle or cardiac microsomes (Fig. 2d,e). The antibodies used for the detection of these two proteins are directed against skeletal muscle isoforms. Triadin and sarcalumenin exist also as heart isoforms [48,49], but since antibodies to both cardiac proteins are not available to us, we could not investigate the expression pattern of these muscle membrane proteins.

Immunoblot analysis of chronic low-frequency stimulated tibialis anterior muscle showed that concomitant with a drastic decrease in the expression of the RyR1 isoform (Fig. 2a), the cardiac-type RyR2 Ca^{2+}-release channel is up-regulated (Fig. 2b). However, in contrast to the amount of RyR2 present in cardiac microsomes, the immunodecoration of this isoform is relatively weak following 4 and 30 days of electro-stimulation. This study shows for the first time that on the protein level the decrease in the skeletal muscle isoform is compensated by an up-regulation of the cardiac isoform of the sarcoplasmic reticulum ryanodine receptor during the fast-to-slow muscle transformation process. Chronic conditioning of fast muscle fibers caused a slight up-regulation of FKBP12 after 30 days of stimulation (Fig. 3c). Furthermore, electro-stimulation had a profound effect on both triad markers of 90 kDa and 94 kDa. Both membrane proteins were drastically down-regulated during the transition process and the relative abundance of both proteins was too low to detect these components in muscle fibers stimulated for more than 4 days (Fig. 2d,e).

3.3. *Expression pattern of Ca^{2+}-reservoir proteins in muscle tissues*

The relative abundance of the major luminal Ca^{2+}-binding proteins of the longitudinal tubules and the terminal cisternae were investigated. Antibodies to calsequestrin of apparent 63 kDa, calsequestrin-like proteins of approximately 120 kDa to 200 kDa, sarcalumenin of apparent 160 kDa and calreticulin of approximately 60 kDa were employed to determine the amount and isoform expression pattern of these sarcoplasmic reticulum components which form the major Ca^{2+}-reservoir of muscle fibers. Calsequestrin exists as a fast fCSQ-isoform and as a slow/cardiac sCSQ-isoform. Monoclonal antibody $VIIID1_2$ labeled the calsequestrin monomer of 63 kDa strongly in fast-twitch muscles and slightly less intensely in slow and neonatal muscles (Fig. 3a). No fast isoform was detected in heart microsomes (Fig. 3a). In mature skeletal muscle, this antibody also recognized the so-called calsequestrin-like proteins [38], which are absent from neonatal muscle fibers (Fig. 3a). In contrast, the slow isoform of calsequestrin was found in all muscle types, whereby the highest abundance was detectable in heart and neonatal muscle (Fig. 3b). The broader immunodecorated bands of higher molecular mass in cardiac and neonatal preparations possibly represent, in analogy to the mature skeletal muscle isoforms, slow/cardiac calsequestrin-like proteins. Immunoblotting with antibodies to calreticulin, a ubiquitous Ca^{2+}-storage protein, revealed it to be present in high amounts in heart and neonatal muscle, but also at lower levels in mature skeletal

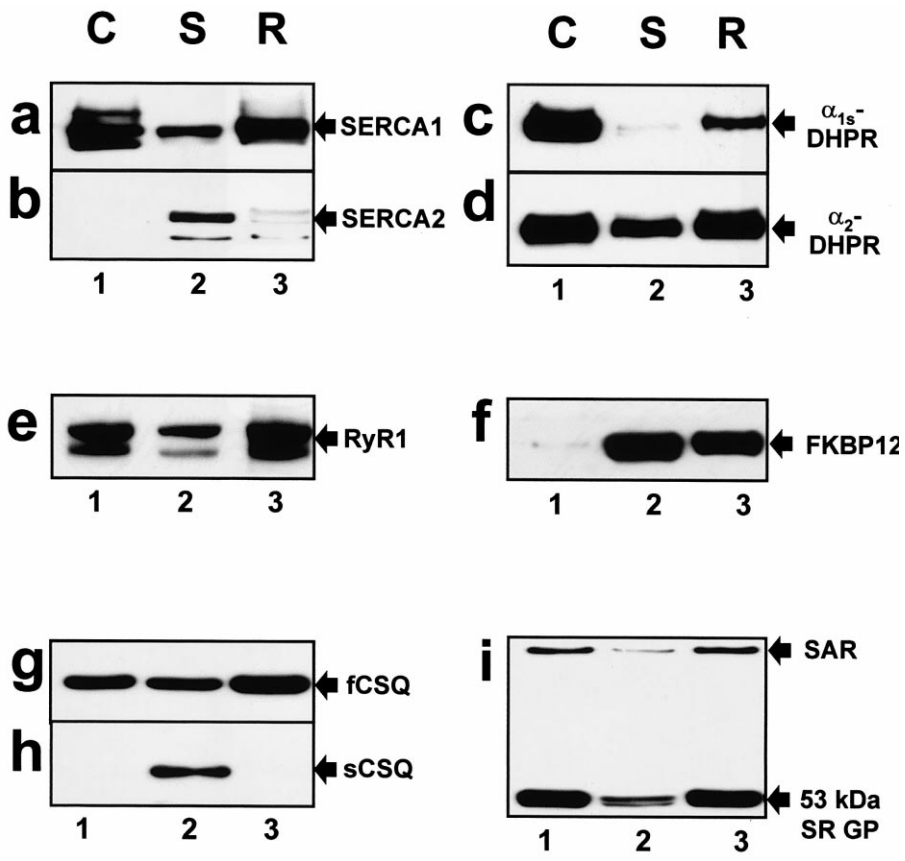


Fig. 6. Recovery study of chronic low-frequency stimulated fast muscle. Shown are immunoblots stained with antibodies to the fast SERCA1 isoform of the sarcoplasmic reticulum Ca^{2+}-ATPase (a), the slow/cardiac SERCA2 isoform of the sarcoplasmic reticulum Ca^{2+}-ATPase (b), the α_{1S}-subunit of the dihydropyridine receptor (α_{1S}-DHPR) (c), the α_2-subunit of the dihydropyridine receptor (α_2-DHPR) (d), the RyR1 isoform of the ryanodine receptor Ca^{2+}-release channel (e), the immunophilin FK506-binding protein of apparent 12 kDa (FKBP12) (c), the fast isoform of calsequestrin (fCSQ) (g), the slow/cardiac isoform of calsequestrin (sCSQ) (h), and sarculumenin (SAR) and its immunologically related 53 kDa sarcoplasmic reticulum glycoprotein (53 kDa SR GP) (i). See Section 2 and Table 1 for a detailed description of antibodies used. Lanes 1–3 represent the microsomal fraction isolated from unstimulated control tibialis anterior muscle (C), 14 days chronic low-frequency stimulated tibialis anterior muscle (S) and tibialis anterior muscle left to recover for 30 days without stimulation following 14 days of chronic conditioning (R), respectively. Individual proteins are marked by arrows on the right.

muscles (Fig. 3c). The major Ca^{2+}-binding protein and luminal Ca^{2+}-transporter in the longitudinal tubules of the sarcoplasmic reticulum, sarcalumenin, and its immunologically related sarcoplasmic reticulum glycoprotein of apparent 53 kDa, were also detectable in all muscle types studied. Their expression was highest in fast muscle and lowest in cardiac and neonatal skeletal muscle (Fig. 3d).

During the fast-to-slow muscle transformation process, the fast isoform of calsequestrin was shown to be present at lower amounts in 30 days stimulated muscle fibers (Fig. 3a), while the slow/cardiac isoform was clearly up-regulated after 4 days of conditioning, with a subsequent decrease in expression at the last time point of stimulation investigated (Fig. 3b). In contrast to unstimulated mature skeletal muscle, potential high-molecular-mass isoforms of slow/cardiac calsequestrin were also present in electro-stimulated samples (Fig. 3b). Chronic low-frequency stimulation had only a slightly elevating effect on the expression of calreticulin in tibialis anterior muscle (Fig. 3c). The longitudinal Ca^{2+}-binding protein sarcalumenin, and the immunologically related sarcoplasmic reticulum glycoprotein of apparent 53 kDa glycoprotein, were both down-regulated following 30 days of stimulation (Fig. 3d).

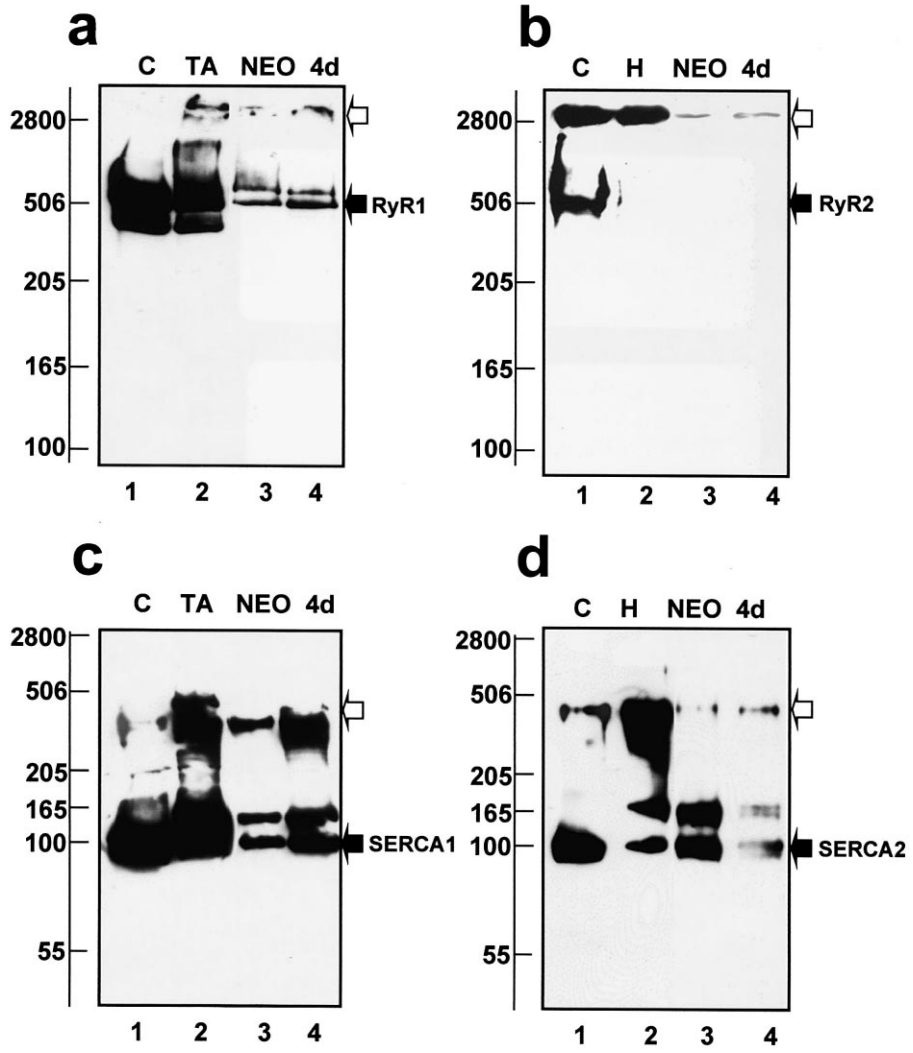


Fig. 7. Chemical crosslinking analysis of the ryanodine receptor and the Ca^{2+}-ATPase. Shown are immunoblots stained with antibodies to the RyR1 (a) and RyR2 (b) isoforms of the ryanodine receptor Ca^{2+}-release channel, as well as the SERCA1 (c) and SERCA2 (d) isoforms of the sarcoplasmic reticulum Ca^{2+}-ATPase. See Section 2 and Table 1 for a detailed description of antibodies used. Lanes 1–4 represent in (a) and (c) microsomes isolated from control tibialis anterior muscle (C), as well as chemically crosslinked membranes from tibialis anterior (TA), neonatal skeletal muscle (NEO) and 4 days chronic low-frequency stimulated tibialis anterior muscle (4d), respectively. In immunoblots (b) and (d), lanes 1–4 represent microsomes isolated from control cardiac muscle (C), as well as chemically crosslinked heart membranes (H), neonatal skeletal muscle (NEO) and 4 days chronic low-frequency stimulated tibialis anterior muscle (4d), respectively. Chemical crosslinking was performed with 100 μg dithiobis-succinimidyl-propionate per mg membrane protein. Molecular weight markers are indicated on the left. Monomers and their high-molecular-mass oligomers are marked by closed and open arrows, respectively.

3.4. *Expression pattern of ion-regulatory membrane proteins in muscle tissues*

Many different ion pumps and ion exchangers are directly or indirectly involved in the regulation of Ca^{2+}-homeostasis in muscle fibers. Since differences exist in the physiological organization of Ca^{2+}-uptake mechanisms between fast muscle, slow muscle and the heart, we investigated the amount and isoform expression pattern of the two major muscle Ca^{2+}-ATPases of the sarcoplasmic reticulum, SERCA1 and SERCA2, as well as two important ion-regulatory surface proteins, the Na^+/Ca^{2+}-exchanger and the Na^+/K^+-ATPase. Immunolabeling of the

SERCA1 Ca^{2+}-ATPase by monoclonal antibody IIH11 clearly revealed the differential expression of this fast isoform between fast-twitch and slow-twitch skeletal muscles (Fig. 4a). The opposite immunodecoration pattern was achieved with monoclonal antibody IID8 against the SERCA2 Ca^{2+}-ATPase, which was found to be predominantly expressed in slow-twitching soleus muscle and the heart (Fig. 4b). Expression of phospolamban, a regulatory subunit of the slow/cardiac Ca^{2+}-ATPase, was also almost exclusively found in slow-twitch skeletal muscle and cardiac muscle (Fig. 4c). Both Ca^{2+}-ATPase isoforms were present at relatively comparable amounts in neonatal skeletal muscle preparations (Fig. 4a,b).

The mature Na^+/Ca^{2+}-exchanger of approximately 180 kDa and its proteolytic fragment of apparent 70 kDa [40] were expressed in all muscle samples investigated with the highest amount in heart, medium levels in slow muscle and lower amounts in neonatal and mature fast muscles (Fig. 4d). In order to properly determine potential differences in the expression levels of this protein, equal amounts of membrane protein from the different types of muscles had to be loaded in individual gel lanes. In addition, a reliable intensity of immunolabeling had to be achieved for fractions with a relatively low density of the Na^+/Ca^{2+}-exchanger. This, however, resulted in a broadening of the immunodecorated band representing the cardiac Na^+/Ca^{2+}-exchanger and can be accounted for by the high amount of this surface protein present in heart as compared to the other muscle fibers investigated. Monoclonal antibody C464.6 to the α_1-subunit of the Na^+/K^+-ATPase labeled all microsomal muscle preparations. This surface protein appears to be expressed in higher abundance in heart as compared to skeletal muscle (Fig. 4e).

Following chronic low-frequency stimulation, a switch in the major isoforms of the sarcoplasmic reticulum Ca^{2+}-ATPase seems to occur. While the expression of the SERCA2 isoform is elevated in 30 days stimulated muscle fibers, the SERCA1 protein species is clearly down-regulated. In analogy to the increase in the SERCA2 Ca^{2+}-ATPase, its regulatory subunit phospholamban is also up-regulated during the fast-to-slow transition process. Interestingly, conditioning of fast muscle fibers also caused an increase in the Na^+/Ca^{2+}-exchanger (Fig. 4d) and the Na^+/K^+-ATPase (Fig. 4e). The expression of the Na^+/K^+-ATPase is higher in 4 days stimulated muscle as compared to 30 days of electro-stimulation (Fig. 4e). A coordinated increase in both ion-regulatory proteins is a very important finding with respect to understanding the physiology of compensatory mechanisms to counteract certain stimulation-induced changes in K^+- and Ca^{2+}-homeostasis.

3.5. *Expression pattern of fast and slow myosin heavy chains in muscle tissues*

For comparative purposes and in order to evaluate the electrophoretically transferred microsomal samples from various muscle types, immunoblotting of myosin heavy chain isoforms was performed. These results reflect the distribution of well established slow and fast markers of the contractile apparatus and can thus be used as an internal standard for the immunoblots of Figs. 1–4. It is well established that chronic low-frequency stimulation induces a stepwise isoform exchange of myofibrilliar proteins [6]. We recently documented the gradual replacement of myosin heavy chains in rabbit tibialis anterior in the following sequence: MHCIIb to MHCIId(x) to MHCIIa to MHCI [12,50]. To establish this transformation pattern in the conditioned muscle specimens studied in this report and to compare it to the myosin expression pattern in the other muscle types investigated, we used monoclonal antibodies MY-33 and NOQ7.5-4D to the fast and slow myosin heavy chain, respectively. Although both antibodies do not exhibit cross-reactivity between their particular immunological determinant, i.e. the fast versus the slow isoform, the antibody directed against the fast isoform does not differentiate between the various fast-twitch species of the myosin heavy chain.

As illustrated in Fig. 5, the application of both antibodies resulted in a clear distinction between the various muscle types. The fast isoform of the myosin heavy chain was found in high amounts in predominantly fast-twitching tibialis anterior and extensor digitorum longus muscles and at a very low level in slow-twitch soleus muscles (Fig. 5a). The opposite expression pattern was observed for the slow myosin heavy chain, which exhibited its highest expression in slow muscle fibers (Fig. 5b). Microsomes isolated from neonatal skeletal muscles contained approximately equal amounts of fast and slow

isoforms, while only the slow myosin heavy chain was expressed in heart (Fig. 5a,b). Chronic electro-stimulation changed the expression pattern of these markers of the contractile apparatus in a predictable way. Parallel with a down-regulation of the fast isoform, an up-regulation of the slow isoform was observed (Fig. 5a,b). This switch in the major myosin heavy chain isoforms established the successful transformation process in the muscle specimens investigated.

3.6. Recovery study of chronic low-frequency stimulated fast muscle

Although an enormous amount of information is available about the reciprocal changes in muscle isoforms in response to the initiation and cessation of chronic electro-stimulation [6,51], relatively little is known about the effect of reversal of chronic conditioning on the expression of Ca^{2+}-regulatory membrane proteins. We therefore performed a recovery study of chronic low-frequency stimulated fast muscle. Following electro-stimulation for 14 days, conditioning was terminated and the muscle left to recover for 30 days prior to immunoblot analysis. The stimulation-induced down-regulation of the SERCA1 isoform of the sarcoplasmic reticulum Ca^{2+}-ATPase (Fig. 6a), the α_{1S}-subunit and the α_2-subunit of the dihydropyridine receptor (Fig. 6c,d), the RyR1 isoform of the Ca^{2+}-release channel (Fig. 6e), the fCSQ-isoform of calsequestrin (Fig. 6g), as well as sarcalumenin and its immunologically related sarcoplasmic reticulum glycoprotein of apparent 53 kDa (Fig. 6i) was partially or totally reversed during the recovery period. In analogy, the up-regulation of the SERCA2 isoform of the sarcoplasmic reticulum Ca^{2+}-ATPase (Fig. 6b) and the sCSQ-isoform of calsequestrin (Fig. 6h) was also reversed following the change to a non-stimulation protocol. Of the above proteins, the reversal of the down-regulation of the α_{1S}-subunit of the dihydropyridine receptor resulted in the lowest levels of recovery as compared to the concentration of these Ca^{2+}-regulatory proteins in normal tibialis anterior muscles. The only stimulation-induced effect, which was not reversed in a major way, was the drastic up-regulation of the FKBP12 protein (Fig. 6f). The analysis of other Ca^{2+}-regulatory proteins during the recovery period did not result in reliable immunodecoration for proper interpretation (not shown) and was thus not further pursued.

3.7. Chemical crosslinking of the ryanodine receptor and the Ca^{2+}-ATPase

The ability to form oligomeric structures under native conditions appears to be an intrinsic property of many Ca^{2+}-regulatory membrane proteins in muscle [9]. In order to determine potential variations in protein–protein interactions between different types of muscle, we carried out a chemical crosslinking analysis. Using optimized concentration ratios between the crosslinking probe and membrane proteins, as well as established conditions with respect to temperature, pH and incubation time [52], we were able to analyze the oligomerization of relatively abundant microsomal proteins. On the other hand, comparative immunoblotting of many less abundant Ca^{2+}-regulatory proteins did not result in good enough labeling for a proper interpretation of shifts in relative electrophoretic mobility (not shown). Nevertheless, we could determine oligomerization of two important muscle membrane proteins which exist as two distinct isoforms, the ryanodine receptor Ca^{2+}-release channel and the sarcoplasmic reticulum Ca^{2+}-ATPase.

Fig. 7 compares crosslinking-induced complexes in adult tibialis anterior muscle, cardiac muscle, neonatal skeletal muscle and 4 days chronic low-frequency stimulated fast muscle. In adult skeletal muscle and heart, the RyR1 and the RyR2 isoform exhibit shifts to high-molecular-mass complexes, respectively, following incubation with the hydrophobic 12 Å crosslinker dithiobis-succinimidyl-propionate (Fig. 7a,b). Using 100 μg crosslinker per mg membrane protein, almost all of the cardiac isoform shifts to a complex of approximately 3000 kDa, while only a sub-population of the major skeletal muscle Ca^{2+}-release channel forms large complexes under these conditions. A similar result was obtained for neonatal skeletal muscle and electro-stimulated fast muscle, but the abundance of both receptors was much lower as compared to the adult or non-stimulated muscle tissues (Fig. 7a,b). Thus, the existence of oligomeric structures of the ryanodine receptor was established in all muscle types investigated.

The chemical crosslinking analysis of the SERCA1 and SERCA2 isoforms of the sarcoplasmic reticulum Ca^{2+}-ATPase in adult tibialis anterior muscle, cardiac muscle, neonatal skeletal muscle and 4 days chronic low-frequency stimulated fast muscle revealed that these microsomal proteins also tend to form high-molecular-mass complexes (Fig. 7c,d). Although the results from the immunoblot analysis of the Ca^{2+}-pumps were less clear than those presented for the ryanodine receptor isoforms (Fig. 7a,b), they revealed crosslinker-stabilized SERCA1 and SERCA2 complexes, estimated to be tetramers of approximately 450 kDa (Fig. 7c,d). Despite chemically reducing conditions, even non-crosslinked Ca^{2+}-ATPase units exhibit a certain degree of oligomeric structures making the interpretation of these findings more difficult. Overall, the crosslinking analysis of the two Ca^{2+}-pump isoforms suggests that, independent of the developmental stage, fiber type or innervation pattern, these relaxation-facilitating membrane proteins exist under native conditions in large aggregates.

4. Discussion

Although differences in the abundance and expression pattern of many muscle-specific proteins have been documented [1], very little is known about the expression of cardiac isoforms of Ca^{2+}-channels and auxiliary proteins on the protein level in conditioned muscle fibers. Here, we compared the expression profile of the major Ca^{2+}-regulatory membrane proteins involved in excitation–contraction coupling, relaxation and triad stabilization in a single study using extensive immunoblotting. In contrast to previous studies on the single cell level [5], the major aim of this investigation was to determine global changes in distinct protein species in the entire fiber population of a specific muscle. Since besides trans-differentiation also degeneration–regeneration processes contribute to the fast-to-slow transition process [53,54], our biochemical approach did not differentiate between different mechanisms associated with phenotypic alterations but analyzed overall changes in protein isoform expression.

The dihydropyridine receptor is a multimeric receptor which consists of the main α_1-subunit and its auxiliary α_2/δ-, β-, and γ-subunits [23]. The α_1-subunits acts as a voltage sensor and/or a Ca^{2+}-channel, depending on the isoform present in the muscle [17]. Since mRNA studies have demonstrated the presence of the α_{1C}-subunit of the dihydropyridine receptor in soleus and diaphragm, it was suggested that these muscle fiber types exhibit a degree of cardiac-like excitation–contraction coupling [14]. In analogy, our immunoblotting data suggest that early functional adaptations to chronic low-frequency stimulation may be accompanied by a switch from skeletal muscle to neonatal/cardiac-like excitation–contraction coupling or the coexistence of both main signal transduction mechanisms in conditioned fibers. We find here that, besides a switch in fast and slow isoforms of the myosin heavy chain, a similar change occurs for the dihydropyridine receptor. The α_{1S}-isoform is drastically decreased and the α_{1C}-isoform increases in abundance in chronic low-frequency stimulated fast skeletal muscles. Because the amount of the α_{1C}-subunit of the dihydropyridine receptor in 4 days electro-stimulated muscle and heart is comparable, the presence of the cardiac isoform could be sufficient for a cardiac-like mode of excitation–contraction coupling [55]. In addition, the increase in this isoform might be also due to the appearance of satellite cell-derived myotubes during degeneration–regeneration processes in the early stages of muscle transformation [53,54].

While the α_1-subunit operates as the principal receptor subunit, the α_2/δ-, β-, and γ-subunits modulate the activity of the α_1-subunit [56,57]. Postnatal studies on rat myotubes showed an early increase of β-subunits prior to the up-regulation of the α_1- and α_2-subunits of the transverse-tubular receptor complex [20]. This suggests that the β-subunit plays a significant role in increasing dihydropyridine receptor numbers during early stages of muscle development. Our immunoblot analysis demonstrated that the β-subunit is strongly up-regulated after 4 days of stimulation but returns to lower expression levels after 30 days. This transient increase of the β-subunit coincided with an increase in the α_{1C}-isoform. Hence, the β-subunit may increase the number of α_{1C}-subunits of the dihydropyridine receptor during physiological adaptations in the fast-to-slow transition process.

In mature skeletal muscle fibers, direct physical

coupling between the voltage sensor of the transverse tubules and the Ca^{2+}-release channel of the sarcoplasmic reticulum mediates signal transduction at the triad junction [58]. Besides electron microscopical evidence [59], co-immuno-precipitation data [60] and chemical crosslinking studies [36], receptor domain-binding experiments [15] strongly support this hypothesis. Relatively short stretches of primary sequence within the II–III loop domain of the α_{1S}-subunit of the dihydropyridine receptor [61] appear to directly interact with a cytoplasmic region of the junctional Ca^{2+}-release channel [62]. The family of intracellular ryanodine receptor Ca^{2+}-release channels are expressed in mammalian tissues as three isoforms: RyR1, RyR2 and RyR3 [63]. Skeletal muscle and the heart contain predominantly RyR1 and RyR2, respectively. The RyR3 isoform has a wider tissue distribution, although originally identified in brain [64]. Our comparative immunoblot analysis confirmed the general expression pattern of the RyR1 and RyR2 isoforms. Previous studies localized RyR3 in brain, diaphragm and slow-twitch skeletal muscle [65]. However, the polyclonal antibody to RyR3 used in this report did not recognize this receptor in the various muscle types examined. This is most likely due to the minuscule amounts of RyR3 present in crude microsomal preparations derived from muscle homogenates, since RyR3 exhibits a several-fold lower abundance in mature mammalian skeletal muscle as compared to the major RyR1 isoform [65].

The expression of ryanodine receptor isoforms undergoes several changes in developing skeletal muscle, whereby both RyR1 and RyR3 are expressed at birth [24]. Although an earlier study described the lack of mRNA encoding for the RyR2 isoform in myotubes [20], our immunoblot data presented here clearly detected this isoform in neonatal muscle specimens 5 days after birth. Hence, RyR2 is expressed in developing skeletal muscle fibers, and this finding further supports the idea of a cardiac-like mode of excitation–contraction coupling in early neonatal muscles. Interestingly, in parallel to an increase in the α_{1C}-subunit of the dihydropyridine receptor, RyR2 is also up-regulated during stimulation-induced adaptations to changed functional demands. This coordinated up-regulation of cardiac isoforms, in parallel with a decrease in fast skeletal muscle receptors, indicates that during the early stages of muscle transformation a neonatal/cardiac-like interaction might occur between the α_1-subunit of the dihydropyridine receptor and the ryanodine receptor complex. Besides true trans-differentiation, degeneration–regeneration mechanisms [53,54] probably also account for some of the observed receptor isoform switching.

The physiological role of many other triad-specific membrane proteins has not yet been fully elucidated. Two junctional sarcoplasmic reticulum elements, skeletal muscle triadin of 94 kDa and another triad marker of apparent 90 kDa, are possibly involved in the regulation of overall Ca^{2+}-homeostasis and/or the structural maintenance of triads [66,67]. Since both proteins are drastically down-regulated during the fast-to-slow transition process, and are absent or present at only very low levels in neonatal muscle and the heart, these isoforms are possibly involved in regulating and/or stabilizing the skeletal muscle mode of excitation–contraction coupling, only. Therefore these two triad markers might directly facilitate receptor coupling in adult skeletal muscle. In contrast, the immunophilin protein of apparent 12 kDa, which binds the immunosuppressant drug FK506 with high affinity, was found at increased levels in heart and 30 days stimulated fast muscle fibers. Consequently, the expression of the overall population of FKBP12 proteins does not seem to be correlated with the expression of the RyR1 isoform of the junctional Ca^{2+}-release channel complex. This ubiquitous protein must therefore be a multifunctional component, which agrees with reports that FKBP12 is also associated with several other proteins including IP_3-sensitive Ca^{2+}-channels [68].

The two major Ca^{2+}-binding proteins of the longitudinal tubules and the terminal cisternae of the highly developed endoplasmic reticulum in skeletal muscle are calsequestrin and sarculumenin, respectively [26,27]. In parallel to a slight down-regulation of fast calsequestrin and sarcalumenin following 30 days of electro-stimulation, the slow/cardiac sCSQ-isoforms of calsequestrin and calreticulin were increased in their relative density in microsomal preparations. Although the sCSQ-isoform is a major element of the cardiac Ca^{2+}-regulatory machinery, in mature slow-twitch muscle of rabbits it accounts for only 25% of the total population of CSQ clusters

[26]. Since over-expression of cardiac/slow calsequestrin in transgenic mice has shown that this luminal component of the sarcoplasmic reticulum is both a storage and regulatory protein in the cardiac muscle Ca^{2+}-signaling cascade [69], our finding would agree with the above discussed stimulation-induced switch to a certain degree of the cardiac type of excitation–contraction coupling. The ubiquitous Ca^{2+}-binding protein calreticulin is not only involved in Ca^{2+}-storage, but has also been implicated in many diverse functions such as chaperone activity, cell adhesion and cardiac development [70]. Functional studies have suggested that calreticulin interacts with the SERCA2 isoform of the sarcoplasmic reticulum Ca^{2+}-ATPase [71]. Calreticulin is drastically increased in its expression following heat shock treatment [72]. Hence, calreticulin may act at least partially as a chaperone during the early stages of muscle transformation. In addition, invasion of mononucleated cells in stimulated muscle [53,54] might account at least partially for the observed up-regulation in this Ca^{2+}-binding protein.

To prevent refractoriness of the Ca^{2+}-regulatory apparatus, following the transient opening of the Ca^{2+}-release units and subsequent triggering of filament sliding, Ca^{2+}-ions are quickly removed from the cytosol by two main physiological mechanisms. In mature skeletal muscle fibers, the major Ca^{2+}-uptake into the sarcoplasmic reticulum occurs via ATPase-driven Ca^{2+}-pumps of the SERCA sub-type [31]. In addition, in cardiac muscle, the surface Na^+/Ca^{2+}-exchanger, indirectly driven by the energy-dependent action of the Na^+/K^+-ATPase, also facilitates fiber relaxation [33]. Our study confirmed the established isoform expression pattern of these Ca^{2+}-regulatory components. The SR Ca^{2+}-ATPase exists in adult muscle tissues as two isoforms, SERCA1 in fast-twitch skeletal muscle and SERCA2 in slow-twitch muscle and the heart. Neonatal muscle is an exception, because both isoforms exhibit comparable expression levels during early postnatal development, but at later stages the SERCA1 Ca^{2+}-ATPase clearly increases during myogenesis [21]. The isoform expression patterns of SERCA1 and SERCA2 were strongly affected by chronic low-frequency stimulation. The switching of the fast isoform to the SERCA2 species was previously shown to be accompanied by a decrease in overall Ca^{2+}-ATPase activity [73]. This enzyme inactivation is only partially due to isoform transitions, but can also be attributed to a partial inactivation of the Ca^{2+}-pump [74,75].

The up-regulation of phospholamban and the Na^+/Ca^{2+}-exchanger in stimulated fast muscle is another strong indication that a heart muscle-like Ca^{2+}-regulatory apparatus is expressed during fiber transitions and degeneration–regeneration processes. Phospholamban, a phosphorylatable regulator of the slow/cardiac Ca^{2+}-pump, is normally only expressed in cardiac and slow-twitch muscle. Our detection of considerable amounts of this protein in electro-stimulated tibialis anterior aggrees with a report by Hu et al. [76], who described a coordinated up-regulation of phospholamban and the SERCA2 isoform in electro-stimulated canine *latissimus dorsi* muscle. Since the Na^+/Ca^{2+}-exchanger is the major pathway for Ca^{2+}-removal from the cytosol in cardiac muscle, it is likely that a switch from a skeletal muscle to a more cardiac-like mode of Ca^{2+}-sequestration occurs following muscle conditioning. The stimulation-induced increase in the Na^+/K^+-ATPase might on the one hand provide the driving force for the up-regulated Na^+/Ca^{2+}-exchanger units, and may on the other hand counteract the loss of K^+-ions. Lack of this cation appears to trigger the transient refractoriness observed in a large population of chronically stimulated muscle fibers during the early phases of the fast-to-slow transition process [11].

Since protein–protein interactions play an important role in the Ca^{2+}-regulatory membrane system of mature muscle fibers [9], the existence of high-molecular-mass complexes was studied by chemical crosslinking. Previous findings that triadic Ca^{2+}-channels and sarcoplasmic reticulum Ca^{2+}-ATPases from normal and stimulated skeletal muscles form very large complexes [9,12,36,77] were confirmed. As reviewed by Martonosi [78], oligomerization of the native SR Ca^{2+}-pump is postulated to provide protection against proteolytic degradation and might also be important for co-operative kinetics between individual subunits within the physiologically active enzyme complexes. Our crosslinking analysis agrees with electron microscopical freeze-fracture analysis which suggests SERCA1 tetramerization in mature skeletal muscle fibers [78]. Since the present study also demonstrated a certain degree of oligomerization of the slow/cardiac SERCA2 isoform in developing and

transformed skeletal muscle, as well as cardiac tissue, one can assume that SERCA protein–protein interactions also play a role during myogenesis and in the heart. In addition, we could show for the first time that the RyR2 isoform of the Ca^{2+}-release channel also forms aggregates in native membranes isolated from heart and electro-stimulated fast muscle. Therefore the majority of Ca^{2+}-regulatory membrane proteins, independent of their tissue distribution or transition stage, seem to have a strong tendency to oligomerize. Direct protein interactions appear to mediate the molecular basis for signal transduction, maintenance of receptor coupling, protein stabilization, co-operative kinetics, and protection against proteolytic degradation in many different kinds of muscle cells [9].

In conclusion, our extensive immunoblot analysis of key components of excitation–contraction coupling and relaxation has clearly shown that the pattern of innervation has a profound influence on the abundance and isoform expression of Ca^{2+}-regulatory membrane proteins in muscle tissues. Specific subsets of isoforms are present in skeletal muscle versus cardiac muscle, developing versus mature skeletal muscle, fast-twitch fibers versus slow-twitch muscles, and unstimulated versus chronic low-frequency stimulated fibers. The diversity in isoform expression suggests that distinct differences exist in the fine-regulation of excitation–contraction coupling, the facilitation of muscle relaxation and the overall storage of luminal Ca^{2+}-ions in the various types of muscle cells. Importantly, since recovery studies demonstrated that stimulation-induced effects on the amount and isoform expression pattern of most investigated Ca^{2+}-regulatory elements are reversible, muscle cells appear to be extremely plastic and are thus highly adaptable to changed functional demands. Biochemical changes in the membrane systems responsible for the regulation of muscle Ca^{2+}-homeostasis reflect physiological adaptations to enhanced contractile activity.

Acknowledgements

This study was supported by project grants from Enterprise Ireland, Dublin (SC/98/241) and the European Community (FMRX-CT960032; QLRT-1999-02034) to K.O. and a travel grant from the European Community Cultural Exchange Program (HEA/DAAD) to K.O., and a research grant from the Deutsche Forschungsgemeinschaft (Sonderforschungsbereich 156, TB/B3) to D.P. The authors wish to thank Dr. Kevin P. Campbell (Howard Hughes Medical Institute, University of Iowa, Iowa City, IA, USA) for his generous gift of monoclonal antibodies used in this study.

References

[1] D. Pette, R.S. Staron, Rev. Physiol. Biochem. Pharmacol. 116 (1990) 1–76.
[2] R.S. Staron, Can. J. Appl. Physiol. 22 (1997) 307–327.
[3] S. Schiaffino, C. Reggiani, J. Appl. Physiol. 77 (1994) 493–501.
[4] R.S. Staron, P. Johnson, Comp. Biochem. Physiol. 106 (1993) 463–475.
[5] D. Pette, H. Peuker, R.S. Staron, Acta Physiol. Scand. 166 (1999) 261–277.
[6] D. Pette, G. Vrbova, Muscle Nerve 22 (1999) 666–677.
[7] D. Pette, R.S. Staron, Int. Rev. Cytol. 170 (1997) 143–223.
[8] W. Melzer, A. Herrmann-Frank, H.C. Lüttgau, Biochim. Biophys. Acta 1241 (1995) 59–116.
[9] B.E. Murray, G.R. Froemming, P.B. Maguire, K. Ohlendieck, Int. J. Mol. Med. 1 (1998) 677–687.
[10] K. Ohlendieck, F.N. Briggs, K.F. Lee, A.W. Wechsler, K.P. Campbell, Eur. J. Biochem. 202 (1991) 739–747.
[11] A. Hicks, K. Ohlendieck, S.O. Goepel, D. Pette, Am. J. Physiol. 273 (1997) 297–305.
[12] K. Ohlendieck, G.R. Froemming, B.E. Murray, P.B. Maguire, E. Leisner, I. Traub, D. Pette, Pflügers Arch. 438 (1999) 700–708.
[13] Y. Pereon, J. Navarro, M. Hamilton, F.W. Booth, P. Palade, Biochem. Biophys. Res. Commun. 235 (1997) 217–222.
[14] Y. Pereon, C. Dettbarn, Y. Lu, K.N. Westhund, J.T. Zhang, P. Palade, Pflügers Arch. 436 (1998) 309–314.
[15] P. Leong, D.H. MacLennan, Biochem. Cell Biol. 76 (1998) 681–694.
[16] M.B. Cannell, C. Soeller, Trends Pharmacol. Sci. 19 (1998) 16–20.
[17] K.G. Beam, T. Tanabe, S. Numa, N.Y. Acad. Sci. 560 (1989) 127–137.
[18] C. Cognard, M. River-Bastide, B. Constanin, G. Raymond, Pflügers Arch. 422 (1992) 207–209.
[19] N. Chaudhari, K. Beam, Dev. Biol. 155 (1993) 507–515.
[20] J. Kyselovic, J.J. Leddy, A. Ray, J. Wigle, B.S. Tuana, J. Biol. Chem. 269 (1994) 21770–21777.
[21] G.R. Froemming, K. Ohlendieck, Biochim. Biophys. Acta 1387 (1998) 226–238.
[22] B.E. Flucher, C. Franzini-Armstrong, Proc. Natl. Acad. Sci. USA 93 (1996) 8101–8106.

[23] W.A. Catterall, Annu. Rev. Biochem. 64 (1995) 493–532.
[24] C. Franzini-Armstrong, F. Protasi, Physiol. Rev. 77 (1997) 699–729.
[25] A.B. Brilliantes, K. Ondrias, A. Scott, E. Kobrinsky, E. Ondriasova, M. Moschella, T. Jayaraman, M. Landers, B. Ehrlich, A.M. Marks, Cell 77 (1994) 513–523.
[26] E. Damiani, P. Volpe, A. Margreth, J. Muscle Res. Cell Motil. 11 (1990) 522–530.
[27] E. Leberer, B.G. Timms, K.P. Campbell, D.H. MacLennan, J. Biol. Chem. 265 (1990) 10118–10124.
[28] M. Michalak, P. Mariani, M. Opas, Biochem. Cell Biol. 76 (1998) 779–785.
[29] G.R. Froemming, D. Pette, K. Ohlendieck, Biochem. Biophys. Res. Commun. 261 (1999) 603–609.
[30] G.R. Froemming, B.E. Murray, K. Ohlendieck, Biochim. Biophys. Acta 1418 (1999) 197–205.
[31] D.H. MacLennan, W.J. Rice, N.M. Green, J. Biol. Chem. 272 (1997) 28815–28818.
[32] D.H. MacLennan, Y. Kimura, T. Toyofuka, N.Y. Acad. Sci. 853 (1998) 31–42.
[33] W.J. Lederer, S. He, S. Luo, W. Dubell, P. Kofuji, R. Kieval, C.F. Neubauer, A. Ruknudin, H. Cheng, M.B. Cannell, T.B. Rogers, D.H. Schulze, N.Y. Acad. Sci. 779 (1996) 7–17.
[34] T. Clausen, Clin. Sci. 95 (1998) 3–17.
[35] K. Naumann, D. Pette, Differentiation 55 (1994) 203–211.
[36] B.E. Murray, K. Ohlendieck, Biochem. J. 324 (1997) 689–696.
[37] E. Harlow, D. Lane, Antibodies. A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1988, pp. 53–138.
[38] P.B. Maguire, F.N. Briggs, N.J. Lennon, K. Ohlendieck, Biochem. Biophys. Res. Commun. 240 (1997) C721–C727.
[39] G.R. Froemming, K. Ohlendieck, Naturwissenschaften 86 (1999) 584–587.
[40] H. Porzig, Z.P. Li, D.A. Nicoll, K.D. Philipson, Am. J. Physiol. 265 (1993) 748–756.
[41] M.A. Movsesian, M. Karimi, K. Green, L.R. Jones, Circulation 90 (1994) 653–657.
[42] T.P. Snutch, W.J. Tomlinson, J.P. Leonard, M.M. Gilbert, Neuron 1 (1991) 45–57.
[43] G. Schwarz, E. Leisner, D. Pette, Pflügers Arch. 398 (1983) 130–133.
[44] U.K. Laemmli, Nature 227 (1970) 680–685.
[45] H. Towbin, T. Staehelin, J. Gordon, Proc. Natl. Acad. Sci. USA 76 (1979) 4350–4354.
[46] S.J. Bradd, M.J. Dunn, Methods Mol. Biol. 19 (1993) 211–218.
[47] S. Barg, J.A. Copello, S. Fleischer, Am. J. Physiol. 41 (1997) C1726–C1733.
[48] Y.M. Kobayashi, L.R. Jones, J. Biol. Chem. 274 (1999) 28660–28668.
[49] N. Hadad, H.E. Meyer, M. Varsanyi, S. Fleischer, V. Shoshan-Barmatz, J. Membr. Biol. 170 (1999) 39–49.
[50] T. Leeuw, D. Pette, Dev. Genet. 19 (1996) 163–168.
[51] C. Brownson, P. Little, J.C. Jarvis, S. Salmons, Muscle Nerve 15 (1992) 694–700.
[52] G. Mattson, E. Conklin, S. Desai, G. Nielander, M.D. Savage, S. Morgensen, Mol. Biol. Rep. 17 (1993) 167–183.
[53] A. Maier, B. Gambke, D. Pette, Cell Tissue Res. 244 (1986) 635–643.
[54] A. Maier, L. Gorza, S. Schaffino, D. Pette, Cell Tissue Res. 254 (1988) 59–68.
[55] T. Tanabe, A. Mikami, S. Numa, K.G. Beam, Nature 344 (1990) 451–453.
[56] L.L. Isom, K.S. De Jong, W.A. Catterall, Neuron 12 (1994) 1183–1194.
[57] C.A. Gurnett, A. DeWaard, K.P. Campbell, Neuron 16 (1996) 431–440.
[58] G. Meissner, X. Lu, Biosci. Rep. 15 (1995) 399–408.
[59] B.A. Block, T. Imagawa, K.P. Campbell, C. Franzini-Armstrong, J. Cell Biol. 107 (1989) 2587–2600.
[60] I. Marty, M. Robert, M. Villaz, K. DeJongh, L. Lai, W.A. Catterall, M. Ronjat, Proc. Natl. Acad. Sci. USA 91 (1994) 2270–2274.
[61] R. El-Hayek, B. Antoniu, J.P. Wang, S.L. Hamilton, N. Ikemoto, J. Biol. Chem. 270 (1995) 22116–22118.
[62] P. Leong, D.H. MacLennan, J. Biol. Chem. 273 (1998) 29958–29964.
[63] Y. Ogawa, N. Kurebayashi, T. Murayama, Adv. Biophys. 36 (1999) 27–64.
[64] P.S. McPherson, K.P. Campbell, J. Biol. Chem. 268 (1993) 19785–19790.
[65] A. Conti, L. Gorza, V. Sorrentiono, Biochem. J. 316 (1996) 19–23.
[66] C.M. Knudsen, K.K. Stang, A.O. Jorgensen, K.P. Campbell, J. Biol. Chem. 268 (1993) 12637–12645.
[67] W. Guo, A.O. Jorgensen, K.P. Campbell, J. Biol. Chem. 269 (1994) 28359–28365.
[68] A.M. Cameron, J.P. Steiner, A.J. Roskams, S.M. Ali, G.V. Ronnett, S.H. Snyder, Cell 83 (1995) 463–472.
[69] L.R. Jones, Y.J. Suzuki, W. Wang, Y.M. Kobayashi, V. Ramesh, C. Franzini-Armstrong, L. Cleemann, M. Morad, J. Clin. Invest. 101 (1998) 1385–1393.
[70] N. Mesaeli, K. Nakamura, E. Zvaritch, P. Dickie, E. Dziak, K.H. Krause, M. Opas, D.H. MacLennan, M. Michalak, J. Cell Biol. 144 (1999) 857–868.
[71] L.M. John, J.D. Lechleiter, P. Camacho, J. Cell Biol. 142 (1998) 963–973.
[72] S.M. Jethmalani, K.J. Henle, J. Cell. Biochem. 69 (1998) 30–43.
[73] E. Leberer, K.T. Haertner, D. Pette, Eur. J. Biochem. 162 (1987) 555–561.
[74] S. Matsushita, D. Pette, Biochem. J. 285 (1992) 303–309.
[75] N. Hämäläinen, D. Pette, J. Muscle Res. Cell. Motil. 18 (1997) 545–554.
[76] P. Hu, C. Yin, K.M. Zhang, L.D. Wright, T.E. Nixon, A.S. Wechsler, J.A. Spratt, F.N. Briggs, J. Biol. Chem. 270 (1995) 11619–11622.
[77] B.E. Murray, K. Ohlendieck, FEBS Lett. 429 (1998) 317–322.
[78] A.N. Martonosi, Biosci. Rep. 15 (1995) 263–282.