Calcium Ion in Skeletal Muscle: Its Crucial Role for Muscle Function, Plasticity, and Disease

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Berchtold, Martin W., Heinrich Brinkmeier, and Markus Müntener. Calcium Ion in Skeletal Muscle: Its Crucial Role for Muscle Function, Plasticity, and Disease. Physiol Rev 80: 1215–1265, 2000.—Mammalian skeletal muscle shows an enormous variability in its functional features such as rate of force production, resistance to fatigue, and energy metabolism, with a wide spectrum from slow aerobic to fast anaerobic physiology. In addition, skeletal muscle exhibits high plasticity that is based on the potential of the muscle fibers to undergo changes of their cytoarchitecture and composition of specific muscle protein isoforms. Adaptive changes of the muscle fibers occur in response to a variety of stimuli such as, e.g., growth and differentiation factors, hormones, nerve signals, or exercise. Additionally, the muscle fibers are arranged in compartments that often function as largely independent muscular subunits. All muscle fibers use Ca²⁺ as their main regulatory and signaling molecule. Therefore, contractile properties of muscle fibers are dependent on the variable expression of proteins involved in Ca²⁺ signaling and handling. Molecular diversity of the main proteins in the Ca²⁺ signaling apparatus (the calcium cycle) largely determines the contraction and relaxation properties of a muscle fiber. The Ca²⁺ signaling apparatus includes 1) the ryanodine receptor that is the sarcoplasmic reticulum Ca²⁺ release channel, 2) the troponin protein complex that mediates the Ca²⁺ effect to the myofibrillar structures leading to contraction, 3) the Ca²⁺ pump responsible for Ca²⁺ reuptake into the sarcoplasmic reticulum, and 4) calsequestrin, the Ca²⁺ storage protein in the sarcoplasmic reticulum. In addition, a multitude of Ca²⁺-binding proteins is present in muscle tissue including parvalbumin, calmodulin, S100 proteins, annexins, sorcin, myosin light chains, α-actinin, calcineurin, and calpain. These Ca²⁺-binding proteins may either exert an important role in Ca²⁺-triggered muscle contraction under certain conditions or modulate other muscle activities such as protein metabolism, differentiation, and growth. Recently, several Ca²⁺ signaling and handling molecules have been shown to be altered in muscle diseases. Functional alterations of Ca²⁺
handling seem to be responsible for the pathophysiologcal conditions seen in dystrophinopathies, Brody’s disease, and malignant hyperthermia. These also underline the importance of the affected molecules for correct muscle performance.

I. INTRODUCTION

The functional units of skeletal muscles are the muscle fibers, long cylindrical multinucleated cells. They vary considerably in their morphological, biochemical, and physiological properties. Different fiber types can be distinguished in each muscle. The fiber type composition, varying from muscle to muscle, is the basis of the well-known structural and functional muscular diversity. The fibers can change their characteristics in response to a large variety of stimuli leading to muscular plasticity. All muscles use Ca²⁺ as their main regulatory and signaling molecule. Therefore, muscle plasticity is closely linked with and highly dependent on the Ca²⁺ handling system.

In 1882 Ringer found that the isolated frog heart contracted when incubated in a solution prepared with London tap water but not in a one prepared with distilled water. This led to the important discovery that the ability of the heart muscle to contract depends on the presence of Ca²⁺ in the external solution (423). Indeed, it has been demonstrated that the function of all muscle types is controlled by Ca²⁺ as a second messenger.

Control of contraction and relaxation by Ca²⁺ in different types of muscle is achieved by three major mechanisms. The first activation mechanism, first discovered and best described, is the troponin-tropomyosin system associated with the actin filaments. It is restricted to skeletal and cardiac muscles. In the second mechanism, found in smooth muscles of vertebrates, Ca²⁺, together with calmodulin (CaM), activates myosin light-chain kinase, which (through phosphorylation of the myosin light chains) initiates muscle contraction. The third mechanism consists of direct binding of Ca²⁺ to myosin which regulates contraction in muscles of certain invertebrates such as scallop. This system depends on the presence of the regulatory light chains of myosin.

The aim of this review is to summarize the present knowledge of muscle plasticity in the context of Ca²⁺ signaling and handling, which is of crucial importance to our understanding of normal muscle function and muscle diseases.

This article focuses mainly on the complexity of the Ca²⁺ handling system in the skeletal muscle of mammals, although reference is made on several occasions to cardiac and smooth muscle and when appropriate to muscles of other vertebrates. Muscle fiber type diversity analyzed at the histological level and functional consequences of fiber type composition are described in some detail (see sect. ii) to emphasize the importance of the morphological studies for understanding muscle plasticity in response to a given stimulus and its dependence on the Ca²⁺ handling apparatus.

Speed of muscle contraction and relaxation as well as other physiological parameters are critically dependent on the special composition of components belonging to the Ca²⁺ handling apparatus. Molecular details of the Ca²⁺ cycle as well as muscle fiber-type specific variations are presented and discussed at a structural and functional level. The main players in the Ca²⁺ cycle are indicated in the schematic presentation shown below.

In the resting state of the myofiber, Ca²⁺ concentrations in the cytosol are maintained at ~50 nM. The Ca²⁺ cycle starts with a surface membrane and transverse tubular (T system) depolarization leading to a release of Ca²⁺ from the sarcoplasmic reticulum (SR) via the ryanodine receptor (RyR), which elevates cytosolic Ca²⁺ locally to ~100 fold higher levels. The conversion of an electrical into a chemical signal at the t-tubule membrane is activated through charge-dependent structural changes of the dihydropyridine receptor (DHPR). Because the DHPR in skeletal muscles are not involved to a major degree in the initial increase of myoplasmic Ca²⁺ as Ca²⁺ channels, they are not discussed in detail. In the skeletal muscle Ca²⁺ binds in a fast reaction to one of the troponin subunits [troponin (Tn) C] on the thin filament. Upon Ca²⁺ binding to TnC, contraction is activated.

In addition to triggering muscle contraction through the troponin system, Ca²⁺ may also affect the muscle contraction apparatus through direct interaction with myosin and other motor proteins. In addition, Ca²⁺ controls the energetics of the muscle by regulating the provision of ATP when needed (512). The latter two aspects are not covered by the present review. Because several transcription factors are known to be regulated by Ca²⁺ (reviewed...
in Ref. 153), another, so far not addressed, possibility for Ca\(^{2+}\)-dependent regulation of muscular activity would be by transcriptional regulation of genes for proteins important in the Ca\(^{2+}\) cycle.

In myofibrils, there is a variety of Ca\(^{2+}\)-binding proteins (CaM, S100 proteins, calpains, calcineurin, sorcin, and annexins) that are not directly involved in the primary process of muscle contraction and relaxation but that may be important for muscle performance and plasticity. Therefore, some features of these proteins (especially CaM and calpain) with respect to their regulatory effects on the primary Ca\(^{2+}\) cycle components are discussed in this review.

Ca\(^{2+}\) translocation from the myofibril to the SR is likely to be facilitated in the fast-twitch skeletal muscle by the high-affinity Ca\(^{2+}\)-binding protein parvalbumin (PV). However, both contraction and relaxation speed decrease as the animal gets bigger. This is mirrored by a decreasing PV content of fast-twitch fibers in larger animals; in humans, these fibers completely lack PV. The energy-dependent Ca\(^{2+}\) uptake into the SR is mediated by the SR ATPase, an enzyme that itself is regulated by both Ca\(^{2+}\) and CaM-dependent phosphorylation. The Ca\(^{2+}\) cycle is completed by binding of Ca\(^{2+}\) to the high-capacity, low-affinity Ca\(^{2+}\)-binding protein calsequestrin.

A major goal of this article is to discuss consequences of malfunctioning of the crucial elements of the Ca\(^{2+}\) cycle that may lead to a variety of muscle diseases. Elevated cytoplasmic Ca\(^{2+}\) levels can cause activation of certain proteases, lipases, and nucleases. Altered physiological properties of muscle, altered gene transcription and transformation of muscle fibers, necrosis, and apoptosis may be consequences. Well-known Ca\(^{2+}\)-related diseases described in this article are certain forms of myopathies (related to channel malfunctioning), malignant hyperthermia (related to Ca\(^{2+}\) release mechanisms), and dystrophinopathies which involve several Ca\(^{2+}\) handling systems and are therefore treated separately in sections II\(D\), II\(B\), and \(v\).

Several recent reviews and book articles deal with various aspects of muscle plasticity and single components of the Ca\(^{2+}\) cycle apparatus. The reader is referred to just one article on each subtopic which should contain sufficient citations for further reading: muscle plasticity (396), myofibrillar protein isoforms (453), molecular muscle diversity (52), the ryanodine receptor (469), the troponin system (490), PV (390), the Ca\(^{2+}\)-ATPase (320), calsequestrin (187), dystrophinopathies (499), calcium release channel diseases (351), and muscular channelopathies (296).

II. SKELETAL MUSCLE AS A DYNAMIC ORGAN

A. Most Muscles Display a Mosaic of Heterogeneous Fiber Types: Compartmental Arrangement

The fiber types present in a muscle can be distinguished with different morphological, biochemical, or physiological methods. Histochemical methods are based mostly on myofibrillar adenosinetriphosphatase (mATPase) activity or on enzymes of the aerobic and anaerobic energy metabolism (Fig. 1).
Morphological and functional differences of muscles and muscle fibers (mainly in frog and rabbit) were known for a long time (170, 412, 481). However, systematic fiber typing did not start until the 1950s after Krüger had distinguished in several vertebrates (including humans) fibers with “Fibrillenstruktur” (evenly dispersed myofibrils) and fibers with “Felderstruktur” (myofibrils arranged in bundles) (273, 274, 276). In the following decades several classification systems were proposed that are summarized in Table 1.

Dubowitz and Pearse (106) described in 1960 histochemically two fiber types, termed type I and type II, respectively. They showed that both fiber types displayed “reciprocal” activities of oxidative and glycolytic enzymes (Fig. 1). Using a histochemical assay for mATPase that was earlier introduced by Padykula and Herman (386), Engel (118) reported a low mATPase activity in the type I and a high mATPase activity in the type II fibers in humans. The group of type II fibers was subsequently subdivided into IIA and IIB fibers (Table 1).

Analyzing the rat semitendinosus muscle by electron microscopical methods, we found that the IIA fibers displayed an intermediate electron density and the IIB fibers showed a high electron density. This agrees with the findings of Engel (118) and may be explained by the “Fibrillenstruktur” of the former and the “Felderstruktur” of the latter fibers. The results of the histochemical and electron microscopical methods were confirmed by the results of the biochemical analyses (412, 461, 546).

In each classification scheme, the different groups are generally nonoverlapping. Although there is a basic correspondence between the different classification schemes, they are not fully interchangeable. Reference numbers are given in parentheses. MHC, myosin heavy chain; mATPase, myofibrillar ATPase.

### 1. Fiber typing

Morphological and functional differences of muscles and muscle fibers (mainly in frog and rabbit) were known for a long time (170, 412, 481). However, systematic fiber typing did not start until the 1950s after Krüger had distinguished in several vertebrates (including humans) fibers with “Fibrillenstruktur” (evenly dispersed myofibrils) and fibers with “Felderstruktur” (myofibrils arranged in bundles) (273, 274, 276). In the following decades several classification systems were proposed that are summarized in Table 1.
microscopy, Gauthier (150) also could distinguish three major fiber types (Table 1). In subsequent ultrastructural and morphometric investigations of mammalian muscles, the classification was mainly based on the mitochondrial content and the width of the Z bands (115, 208, 379). With respect to mitochondria, it should be noted that in small mammals (e.g., mouse, rat) the highest amount of mitochondria is seen in type IIA fibers, whereas in larger animals this is the case with type I fibers. Type IIB fibers exhibit the smallest width of the Z band paralleled by the lowest relative volume density of mitochondria. However, human muscle fibers can more reliably be classified by electron microscopy on the basis of the M-band structure (474).

Schiaffino et al. (455) described in 1985 in the rat an additional fast-twitch fiber type that was later termed type 2X. In the late 1980s, Pette and co-workers (18) electrophoretically identified the myosin heavy chain (MHC) IId in rodents. Fibers containing this MHC isoform were termed type IID fibers. It was shown that type IID fibers are identical to type 2X (IIX) fibers and hence these fibers are designated as type IID/X fibers (Table 1).

In different species more than 10 intermediate fiber types have been described histochromically and biochemically in limb and trunk muscles (14, 46, 226, 231, 243, 251, 304, 433, 492, 494). Most of these subtypes or intermediate types have been shown to be hybrid fibers with respect to the coexistence of different types of myosin. In normal mature muscle fibers of humans and rodents, the coexistence of different slow- and fast-type MHC isoforms is frequently observed (30, 38, 94, 287, 395, 446, 447, 476, 493); all three MHC isoforms I, IIa, and IIb can occasionally be coexpressed in a single muscle fiber (447). With increasing age (see below) also the percentage of muscle fibers coexpressing two or three MHC increases (5).

Some muscles of the craniofacial region which are not concerned with locomotion, such as extraocular (e.g., rat, Ref. 548), laryngeal (e.g., rabbit, thyroarytaenoid, Ref. 312), or jaw-closing muscles (e.g., cat, masseter, temporalis, Ref. 434), exhibit “super-fast” fibers. These fiber types contain superfast MHC isoforms and are phenotypically distinct from both fast-twitch oxidative and fast-twitch glycolytic muscle fibers of the body and limbs.

Presently, for practical reasons, the most widely used classification of fiber types is still the one based on the pH lability of the mATPase activity which distinguishes only type I, IIA, and IIB fibers (46). Unfortunately, in many investigations, no distinction is made between type IIB fibers and the recently discovered IIX fibers (164, 452). The fast fiber types showing the lowest activity of the cytochrome c oxidase (or succinate dehydrogenase, SDH) and the highest activity of the a-glycerophosphate dehydrogenase are also in humans, analogously to rat and mouse, frequently termed IIB fibers. However, in humans, these fibers express the IIX but not the IIB MHC isoforms (119, 446) and should be correctly designated IIX fibers.

Therefore, these fibers are termed in this review IIB when dealing with rat, mouse, or rabbit muscle and IIB(IIX) when dealing with human muscle (Fig. 1). The histochemical staining characteristics of a given fiber type may vary considerably from species to species. The different schemes (Table 1) of classification are not fully interchangeable (79, 370, 488). Only recently simultaneous measurements of mATPase, SDH, and a-glycerophosphate dehydrogenase activities and cross-sectional area in MHC-based fiber types have been performed (427). Significant interrelationships between these parameters have been found on a fiber-to-fiber basis.

2. Metabolic fiber profile and physiological characteristics

The fiber types show differences in their oxidative and glycolytic capacities that generally correlate with differences in contractile and other physiological properties (Table 1). However, these correlations are only general ones. In a histochemically defined fiber type, the metabolic profile and the physiological characteristics need to be defined separately.

Twitch contraction (i.e., time to peak) and half-relaxation times differ to a great extent between fast-twitch and slow-twitch muscle fibers, but there is a substantial overlap between these two groups. These properties are critically dependent on the Ca2+ sensitivity of the contractile apparatus and on the efficiency of Ca2+ uptake into the SR. A third factor is the efficiency of the myosin motor itself, which is composed of different protein isoforms in different muscle fibers. Many events in the Ca2+ cycle also contribute to large differences in resistance to fatigue between fast and slow muscles (reviewed in Ref. 497).

3. Compartmentalization (i.e., a regional specialization of muscle fibers)

Already at the beginning of this century specific regional variations in fiber composition have been noticed in mammalian muscles (96). In the rat, some neck and thoracic (165, 169, 314, 401) and most limb muscles (13, 16, 409, 410, 529) exhibit a predominance of oxidative type I and type IIA fibers in the deep portions and a predominance of glycolytic type IIB fibers in the superficial portions. This is demonstrated with staining for the fast fiber (IIA and IIB) specific PV in the rat extensor digitorum longus muscle (Fig. 2). Lexell et al. (302) found in extensor digitorum longus and tibialis anterior muscle of rabbits an analogous situation. In humans, a similar gradient from deep to superficial has been found in muscles of the upper and lower limb (237, 475), in paravertebral muscles (473), and the masseter muscle (424). Although the trapezius muscle exhibited an increase of type I and IIA fibers at the expense of type IIB (IIX) fibers from...
cranial to caudal in both genders (305, 306), the temporalis muscle showed an increase of the proportion of pure slow type I fibers in a postero-anterior direction at the expense of hybrid slow/fast fibers (268). Quantitative morphological study of whole vastus lateralis muscle from childhood to old age has revealed a life-long rearrangement of these compartments (475).

4. Muscle fiber transformation and type-specific gene expression

Much information is available on the biochemical, morphological, and physiological phenotype of specific fiber types and how these fiber types can be transformed. However, little is known on the accompanying changes in the expression of the corresponding genes and the involved control mechanisms. The signals and early cellular events that exert this control are poorly understood. Several major technical problems hamper the analysis of fiber type specific gene expression. Investigations cannot be carried out on cultured cells, since these do not differentiate to the point they do in vivo. Therefore, work on whole animals has to be carried out. Some data are available from transgenic mouse work. For example, the myosin light chain (MLC) 1 fast (1f) promoter (102) or the MLC3f promoter (249) in combination with the enhancer located 3′ to the MLC1/3 locus (containing both genes) were found to be expressed in fast type II fibers of animals made transgenic with these regulatory elements coupled to reporter genes. This indicates that the used promoter together with the enhancer contain sufficient information to allow fiber-specific expression. However, correct subtype distribution as found in the normal situation was not achieved. A variety of factors may be responsible for this discrepancy. Maybe not all required DNA elements were present in the constructs. Alternatively, the site of transgene integration into the genome, the chromatin configuration or genomic imprinting during embryonic development might have caused the differences.

The method of direct gene transfer into the muscle has been used to investigate the regulatory sequences for the fiber type-specific expression of the MLC-1 slow/ventricular (MLC-1s/v) gene. Constructs with 5′-flanking regions of this gene showed a preferred expression pattern in the slow fibers (534) as found in the endogenous situation. However, several problems are encountered as well when this method is used. The transgenic DNA is not in a genomic configuration, since it is localized on an extrachromosomal plasmid and expression is relatively low if the muscle is not regenerating. So far, no common sequences present in different genes with the same fiber type specificity are known. It is also not established how the known myogenic transcription factors govern fiber type-specific gene expression. There are some indications that members of the myogenic helix-loop-helix transcription factor family such as MyoD, which is expressed mainly in type II fibers, and myogenin, which is expressed mainly in type I fibers (217, 537), are involved in the differentiation and transformation process. However, there is no clear evidence for a specific causal involvement (271). Differentiation of fast-twitch and slow-twitch fibers can also occur when either the MyoD or the myogenin gene is knocked out. Possibly there exists redundancy in transcription factors for muscle differentiation.

FIG. 2. Immunohistochemical demonstration of parvalbumin in the superficial and deep portion of rat extensor digitorum longus muscle. Because parvalbumin is involved in the relaxation process, the type IIB fibers that are fast contracting and fast relaxing show throughout a strong staining intensity. Sixty to seventy percent of the type IIA fibers are intermediately stained, whereas the remaining type IIA and the type I fibers that are slow contracting and slow relaxing are nonreactive. The compartmentation of the muscle is clearly seen; the superficial ("white") portion displays a higher relative amount of IIB fibers than the deep ("red") one. Original magnification, ×40.
Other groups of transcription factors such as MEF-2, M-CAT binding factor, and SRF known to regulate muscle genes have not been investigated for their involvement in fiber type-specific gene expression (453).

Regulation of fiber type-specific gene expression can also be achieved posttranscriptionally. It has been shown, for example, that the pool of transcripts for a muscle gene family producing several isogenes remains constant although levels of the different isogene products may vary greatly during development. This has been demonstrated, for example, for TnC and TnCf isogenes (538).

5. Summary

In conclusion, every muscle within an animal is unique in terms of fiber type composition and distribution pattern within the muscle. In animals the fiber composition of homologous muscles can vary considerably from species to species. Within a species in a given muscle the proportion of type I fibers increases with body size and body weight. In contrast, in humans the interindividual variability of the fiber composition is considerable. Although a given muscle may consist mainly of fast-twitch fibers in one individual, it may be totally made up of slow-twitch fibers in another individual (237, 527, 544, 545). A muscle can functionally adapt to a broad spectrum of activities. The molecular mechanisms involved in these adaptations and the early molecular and cellular events taking place in these processes are still poorly understood. However, DNA sequences important for the regulation of fiber-specific gene expression as well as transcription factors involved in this process can now be investigated by the use of transgenic animals or by direct gene transfer into the muscle of living animals.

B. Changes of Fiber Type Composition and Calcium Handling Apparatus During Development and Aging

1. Development

In vertebrates, most skeletal muscles derive from the paraxial mesodermal tissue that condenses into the segmentally arranged somites. During the further maturation in each somite, the cells are compartmentalized. The dorsolateral compartment is called myotome; it contains two subsets of myogenic precursor cells. The cells of one subset are destined to become the axial musculature, whereas the cells of the other subset migrate into the periphery to form the muscles of the body wall and the limbs (see Refs. 32 and 57 for further references). Myogenic determination occurs independently in somites and limb buds (239). The myogenic precursor cells differentiate to become myoblasts, which later fuse to become three discrete populations of myotubes (first, secondary, tertiary) that then develop into myofibers (104). The later stages of myogenesis are more dependent on the myogenic regulatory factor (MRF) myogenin than early stages (532). Protein and mRNA studies have demonstrated that myosin isoforms follow an embryonic > neonatal > adult transition during mammalian and avian skeletal muscle development (547). In mice, the accumulation of slow MLC in the slow-twitch muscle fibers occurs during prenatal myogenesis, whereas the accumulation of the fast MLC in the fast-twitch muscle fibers is a postnatal phenomenon (541). For further details of embryonic myogenesis and the regulatory pathways underlying the generation of the definitive skeletal muscle diversity, the reader is referred to the following reviews (52, 248).

Studies on chicken muscle have shown that the Ca\(^{2+}\) handling system (Ca\(^{2+}\) release, storage, and uptake) develops in two stages. A temporary Ca\(^{2+}\) regulating system is established at the periphery of the myotubes during myofibrillogenesis (around embryonic day E5.5). This peripheral system is subsequently replaced by the more highly specialized central system (t tubules/SR) during myotube-to-myofiber transition (between E15 and E16) (510). The avian calsequestrin homolog, a Ca\(^{2+}\)-binding protein responsible for Ca\(^{2+}\) storage in the SR, was detected in limb primordia of chicken embryos as early as E5 (67). Calsequestrin and its mRNA increased ~10-fold before myoblast fusion. Cross-linking studies revealed that, during postnatal development, the oligomerization state of Ca\(^{2+}\) regulatory components including the RYR, the sarco(endo)plasmic reticulum Ca\(^{2+}\)-ATPase (SERCA) and calsequestrin increased (143). This indicates that protein-protein interactions become more and more complex during development and are important for the correct function of the adult muscle.

The appearance of PV during myogenesis and maturation has been investigated in frog and rat; in Xenopus laevis, PV is first detected at embryonic stages 24–25, when myotomal muscles are differentiating (463). In the rat, PV immunoactivity appears only postnataally and varies considerably from muscle to muscle. PV can be detected in the tibialis anterior muscle at the fourth postnatal day where it reaches the adult checkerboard pattern 2 days later. In contrast, in the intrinsic muscles of the tongue, in diaphragm, and in intercostal muscles, PV immunoactivity does not appear until the second week. The fact that differences in PV expression do not correlate in time with the differentiation of fiber types (as judged by myosin ATPase activity) probably suggests that myosin and PV are regulated by different mechanisms (384).

2. Aging

In aging rodents, a progressive loss of muscle fibers paralleled by fiber type conversion (see below) from “fast
C. Fiber Transformations and Modulation of Calcium Signaling and Handling Depending on Altered Neuronal Input, Exercise, and Other Factors

1. Neural input, cross-reinnervation, and electrical stimulation

Muscle fiber transformations as the basis of muscular plasticity occur in response to a variety of systemic or local stimuli in humans and animals. Investigation of muscle plasticity mainly started after the classical cross-reinnervation experiments of Buller and co-workers in 1960 (50). Since then it has been repeatedly shown that the firing patterns of the innervating motoneurons largely determine the characteristics of muscle fibers (195) (for further references, see Refs. 52, 396, 397, 440). Thus re-innervation by motoneurons with a different firing pattern leads, within a few months, to changed properties of the reinervated muscles. This is evidenced by an altered fiber type distribution with corresponding changes of the concentration of the fast fiber specific PV (360, 362) and other proteins important for Ca$^{2+}$-handling in the muscle (364). It has been shown that the degree and the time course of the fiber transformation depends on the size ratio of the two muscles which are cross-reinnervated (51, 244). It also depends on the ratio of type IIA and type IIB motoneurons within the reinervating motor nerve (514).

The effects of cross-reinnervation can, to a large extent, be both reproduced and opposed by long-term electrical stimulation (reviewed in Refs. 396, 399, 400). Artificial stimulation that activates all motor units of the stimulated muscle induces a specific remodeling of the muscle fibers leading to a shift of the fiber type distribution. This remodeling encompasses the major, myofibrillar proteins, membrane-bound and soluble proteins involved in Ca$^{2+}$ dynamics, and mitochondrial and cytosolic enzymes of energy metabolism. Stimulation work has been mainly carried out on fast-to-slow transition by chronic low-frequency stimulation (continuous at 10 Hz) (232, 440) and much less on slow-to-fast transition by phasic high-frequency stimulation [e.g., 60 pulses at 100 Hz every 60 s (309, 310) or 40 pulses at 40 Hz every 5 min (301)].

Both types of conversion show substantial species differences that are still not yet fully understood. They involve, e.g., the replacement of degenerating and de novo formation of regenerated fibers in rabbits (460) and guinea pig (301), whereas they are entirely due to transformation of preexisting fibers in rats (91, 301). When rabbit tibialis anterior muscles were stimulated continuously at 2.5, 5, or 10 Hz for 10 Hz, interestingly in muscles that had received 2.5-Hz stimulation, fast myosin isoforms were found to predominate, and the muscles showed the highest levels of oxidative and glycolytic activity (506).

Possible differences in posttranscriptional regulation may result in the transient accumulation of atypical combinations of fast and slow MLC and MHC isoforms, giving rise to the appearance of hybrid fibers (294). Recently, it has been suggested that the drastic depression of the energy state in stimulated muscle fibers could act as an important signal initiating the fast-to-slow transformation process (72). Already after 3 wk of chronic low-frequency stimulation the neuromuscular junctions of the stimulated (fast-twitch) muscles showed a partial transformation toward a morphology characteristic of slow-twitch muscle in rabbits (480). The neuromuscular junctions became smaller, and the secondary postsynaptic folds were more closely spaced. In senescent rats, the fiber shift was significantly less pronounced after low-frequency stimulation (539). This stimulation pattern suppressed the expression of the Ca$^{2+}$-binding protein PV in...
fast-twitch rabbit muscles (259, 260). In humans, intermittent electromyostimulation could increase endurance without concomitant morphological or biochemical changes (253).

2. Physical exercise and detraining

Muscle fiber transformations in consequence of cross-reinnervation or electrical stimulation have been mostly studied in rodents. In humans, the most intensively studied fiber transformations are both the ones following physical exercise and detraining (10, 36, 160). For many years, it has been recognized that endurance training leads to an increase of slow-twitch type I fibers (162, 230, 231). Only much later was it shown conclusively that the fiber distribution can also change in the opposite direction (an increase of fast-twitch type II \([A + B(X)]\) fibers) as a consequence of repeated 30-s “all-out” sprints (120, 121, 229).

3. Overload and hypogravity

Partly comparable with physical exercise and detraining are mechanical overload and hypogravity, respectively. Mechanical overload (induced by stretch or ablation or tenotomy of synergists) leads to hypertrophy. Ultrastructural myofibrillar disruptions, mitochondrial alterations, glycogen pooling, and a significant increase in the number of myonuclei and satellite cells are observed in the early stages (3, 479). Recently, it has been shown that calcineurin plays an important role as a mediator of the \(\text{Ca}^{2+}\) effect on gene transcription in hypertrophy (111, 366, 468; for more details, see sect. ivE). Additionally, fiber splitting paralleled by a shift of the fiber distribution toward the oxidative type I fibers has been reported (179, 247, 391). Many studies show that in several animal species certain forms of mechanical overload can increase muscle fiber number (10, 247). Overload experiments have shown that active musculature not only produces much of the circulating insulin-like growth factor I (IGF-I) but also utilizes most of the IGF-I produced (see review in Ref. 159). The discovery of the locally produced IGF-I and muscle-specific differences were observed in regulation of myosin heavy chain isoforms by thyroid hormones (289). PV distribution and concentration were largely unaffected in all thyroid states. This indicates that the muscular alterations are likely caused by a direct action of the thyroid hormone on muscle fibers, and not via their nervous input (363). The sexually dimorphic muscles (e.g., perineal, masticatory, laryngeal), also under strong hormonal control, will not be further considered.

4. Hormones

Many hormones (e.g., growth hormone, insulin, thyroid hormones, sex hormones) exert a strong systemic influence on skeletal muscles during development as well as in the adult stage (for further references, see Refs. 134, 136). The hormonal effect on muscles is also mirrored in the widespread use and misuse of hormone analogs, e.g., in sports or meat production.

For the thyroid hormones, it has been shown in rats that both hypo- and hyperthyroidism were paralleled by modifications in the fiber type composition. \(3,3',5\)-Triiodothyronine \((\text{T}_3)\) induces terminal muscle differentiation and regulates fiber type composition via direct activation of the muscle-specific myoD gene family (103). Gender- and muscle-specific differences were observed in regulation of myosin heavy chain isoforms by thyroid hormones (289). PV distribution and concentration were largely unaffected in all thyroid states. This indicates that the muscular alterations are likely caused by a direct action of the thyroid hormone on muscle fibers, and not via their nervous input (363). The sexually dimorphic muscles (e.g., perineal, masticatory, laryngeal), also under strong hormonal control, will not be further considered.

5. Unspecific local stimuli

In addition to these specific and/or systemic stimuli, also local and unspecific stimuli can elicit muscular reactions. As an example, fiber transformations of fast- into slow-twitch fibers, and vice versa, have been observed in neck muscles of the rat after an incision of the overlying skin (359).

6. Muscle fiber transformation

In mammalian muscles, fiber transformations probably occur according to the following scheme (modified from Refs. 30, 164, 229, 231, 359)
Endurance exercise or overload, for example, lead to a transformation in the direction from right to left, whereas detraining or hypogravity leads to a transformation in the opposite direction. The fiber type at right is IIB in rodents and IIX in humans (see above). The "intermediate" fiber types are intermediate with respect to metabolic profile and myosin composition. In normal muscles, these fiber types are found in only small amounts. However, in muscles undergoing a transformation, their percentages are increased independently of the direction of the fiber transformation. Such an increase has been shown for type IB and IIC fibers (between type I and IIA in the scheme) in rats after unspecific stimulation (359) and for IIC fibers in healthy humans after physical training (231) or in patients with cervical dysfunctions (527, 545). Subjects with mandibular prognatism and deficient occlusion have revealed in their masseter muscle an increased frequency of intermediate IM fibers (between type I and IIA) (425, 516).

7. Summary

In summary, skeletal muscles are composed of a large variety of morphologically and functionally different fiber types. Today the classification of fiber types based on the pH lability of their mATPase (alkali labile/acid stabile and vice versa) is still widely used. However, to overcome its limitations, fiber types have to be defined according to additional criteria (e.g., analysis of MHC, metabolic profile). The arrangement of the heterogeneous muscle fibers in variable compartments leads to the uniqueness of every muscle. As dynamic structures, muscle fibers are able, although with considerable species differences, to change their morphological and functional characteristics in response to a large spectrum of both local and general stimuli.

D. Fiber Transformation in Diseased Muscles: Hereditary Myotonias and Periodic Paralyses

Muscle fiber transformations paralleled by an altered fiber composition are also encountered as secondary effects in muscle diseases as hereditary myotonias and periodic paralyses, which are disorders of skeletal muscle excitability. Myotonia is caused by runs of nerve independent action potentials at the sarcolemma (Fig. 3, myotonic response). Incomplete muscle relaxation and transient muscle stiffness are the consequences of this hyperexcitability (234, 435). Paralysis is brought about by strong membrane depolarization and following inexcitability of the sarcolemma. Before 1990, the underlying genetic defects were not known in any of these diseases in humans and animals (mouse, goat, and horse). Since that time, most of the disorders have been recognized as mutations in genes coding for voltage-dependent ion channels. Recently, the diseases, called muscular channelopathies, were reclassified and grouped as either sodium channel (SkM1) disorders, chloride channel (ClC-1) disorders (Fig. 3, Table 2), or Ca$^{2+}$ channel disorders. This subject has been extensively reviewed (203, 295, 296). The most frequent disease of this group of disorders is probably the recessive chloride channel myotonia (affected between 1:23,000 to 1:50,000). The aim of this section is to discuss the secondary consequences of increased muscle excitability and activity on muscle structure, function, and fiber type composition in the different affected species.

The hereditary Na$^+$ and Cl$^-$ channelopathies are not accompanied by muscle fiber necrosis, regeneration, or persistent weakness. In some cases of the dominant chloride channel disorder myotonia congenita (Thomsen) muscle fiber hypertrophy, an increased number of central nuclei, and type I fiber atrophy were observed (45), whereas other cases were normal. In recessive myotonia (Becker), also caused by ClC-1 gene mutations, three-quarters of the patients show muscle hypertrophy. A

![Fig. 3. Myotonia and muscle fiber type changes.](http://physrev.physiology.org/)

Mutations in Na$^+$ (1) or Cl$^-$ (2) channels or the lack of the sarcolemmal chloride channel (ClC-1; 2) can lead to overexcitability of the sarcolemma (myotonia). Normal muscle responds with single action potentials upon single stimuli, whereas myotonic muscle often responds with runs of action potentials. Increased membrane excitation can cause protein kinase C (PKC) activation in the nucleus and changes in the pattern of myogenic regulating factors (MRF). The myogenic factors control gene transcription and thereby with couple membrane excitation to the muscle fiber type. A second signaling pathway involves cytoplasmic Ca$^{2+}$. The propagation of action potentials into the transverse tubule system (TT) activates the L-type Ca$^{2+}$ channel and stimulates Ca$^{2+}$ release from the sarcoplasmic reticulum (SR) via theryanodine receptor (RyR). A Ca$^{2+}$ signaling pathway into the nucleus is suggested.
slight increase of serum creatine kinase (CK) was found in some cases. Occasionally, muscle biopsies from patients with paramyotonia congenita, a dominant Na⁺ channel disorder, showed focal myofibrillar damage (149). Myotonia of mouse and goat are caused by chloride channel (ClC-1) defects; however, the phenotype shows differences between the species. In murine myotonia, the degree of muscle stiffness and the frequency of the myo-
tonic discharges are much more pronounced compared with myotonia in humans and goats. Thus myotonic mouse muscle is a biological model of a chronically and extensively stimulated muscle (267, 336, 550). In aged myotonic mice, elongation of tendons and bone deformations have been observed (193). This points to a considerable increase of force development of myotonic muscle in vivo.

1. Alteration of fiber type composition of myotonic muscle

In mice and to a minor extent in the myotonic goat and humans, muscle histological, immunohistochemical, and biochemical investigations revealed secondary changes of myotonia. Reduced glycolytic enzyme activity (482) and changes in the muscular lipid composition (414) were reported. Both findings are consistent with increased amounts of mitochondria in myotonic muscles. In 1984, Jockusch and co-workers (501) showed that in myotonic adr muscle the content of PV was drastically reduced (adr, arrested development of righting response). They suggested that the impaired muscle relaxation seen in adr mice could be a consequence of the PV deficiency. After clarifying that electrically induced myotonia is responsible for the aftercontractions of adr muscle (336), the biochemical changes in myotonic muscle were reinterpreted as fiber type transformations in response to the different stimulation pattern. The most drastic changes occur in predominantly fast-twitch muscle in the myotonic mouse. Electrophoretic and histochemical analysis (234) revealed a shift from IIb to IIa myosin heavy chain. The difference may be explained by the fact that the muscle fiber type composition of bigger mammals differs from that of small rodents by a higher proportion of type I fibers at the expense of type IIA and IIB fibers. In human myotonic muscle, a lack of fast-twitch glycolytic type IIB (IX) fibers was reported (77). This is consistent with the reduction of type IIB fibers in mice and the reduction of the MHCIIb isoform.

In addition to the fiber type changes, muscle hypertrophy was observed in some human disorders with myotonia, independent of whether they are based on Cl⁻ channel (45) or on Na⁺ channel mutations. Our favored interpretation for this finding is that the myotonia is equivalent to exercise and stimulates protein synthesis in muscle. In contrast to human myotonia, mouse myotonia is accompanied by reduced body weight and reduced muscle mass of the affected animals (542). This seems first surprising, but as mentioned above, mouse myotonia is more intense than human and goat myotonia. A reduced opportunity of food intake, insufficient respiration, and other handicaps may be responsible for the growth restriction of myotonic mice.

2. Muscle activity, intracellular signaling, and gene transcription

Fiber type transformation in myotonic muscle and in chronically stimulated muscle has been described at the protein and mRNA levels for many years. However, it is still not clarified how altered muscle membrane and contractile activity influences muscular gene transcription. In the last few years two signal transduction pathways have been discovered that seem to be important for the coupling of membrane excitation and altered gene transcription. First, it was shown by Huang et al. (215) that protein kinase C (PKC) couples membrane excitation to acetylcholine receptor inactivation (Fig. 3). After electrical stimulation of denervated chicken muscle the activity of PKC in the nucleus was found 100-fold increased, and this increase was correlated with the inactivation of AChR subunit genes. Later the same group showed that the myogenin gene, coding for a transcription factor belonging to a family of myogenic factors (48), declined in transcriptional activity after electrical stimulation comparable to the rate of AChR gene inactivation (213). It has been reported that phosphorylation by PKC inactivates myogenin (303). Compared with controls, myotonic adr muscle is characterized by increased levels of the myogenic factors myogenin and herculin (or MRF4) and a reduction of the MyoD level. The differences in mRNA levels of MHCIIb, MHCIIa, MHCIIx, and MHC I genes (158) were attributed to the different pattern of myogenic factors.

The second signal transduction cascade that came into question involves intracellular Ca²⁺ (Fig. 3). Huang and Schmidt (214) showed that electrical stimulation, via an increase of Ca²⁺, causes AChR α-subunit gene inacti-
It has not been clarified whether this mechanism also involves myogenic factors. Recently, it was shown that calcineurin, a calcium-dependent phosphatase, is a possible mediator of fiber type conversion in response to electrical stimulation. The overexpression of calcineurin in cultured muscle caused slow-fiber-specific gene expression, whereas calcineurin inhibition led to a slow to fast conversion of rat soleus muscle in vivo (66) (for further discussion, see sect. IV E). In other cell systems (neurons, glial and liver cells, and T lymphocytes) CaM and CaM-binding proteins have been detected in the nucleus (15). It has further been shown (in nonmuscle cells) that CaM, via the activation of CaM-dependent kinases II or IV, can phosphorylate transcription factors, and it was suggested that CaM may have a general role in RNA processing or splicing (15). Calcium, in most cases together with CaM, can use different routes to signal through ras to modulate survival, differentiation, and plasticity in neurons (130).

3. Summary

The primary defects in myotonias and periodic paralyses are due to mutations in the genes coding for voltage-dependent ion channels. The increased membrane excitation causes several secondary changes including fiber type transformations. The murine animal models of myotonia will be especially valuable in elucidating the linkages between membrane excitation, muscle activity, and gene transcription. Changes in Ca\(^{2+}\)/CaM-dependent cell signaling are likely to be involved in the secondary changes observed in myotonic muscle.

III. PLASTICITY OF THE CALCIUM HANDLING APPARATUS

A. Calcium release from the SR

1. Structural and functional considerations

The RyR to which the plant alkaloid ryanodine specifically binds is the major channel for Ca\(^{2+}\) release from intracellular stores in skeletal muscle; it mediates the t-tubular depolarization-induced Ca\(^{2+}\) release from the SR (Fig. 4). Several review articles exist on the structure and function of the RyR (74, 131, 324, 458). In skeletal muscle, activation of Ca\(^{2+}\) release from the SR is controlled by a voltage sensor in the transverse tubular (tt) membrane (459). Elementary Ca\(^{2+}\) signal events have recently been subcellularly localized in the skeletal muscle. These signals represent openings of individual RyR in the SR membrane and have been termed Ca\(^{2+}\) sparks and Ca\(^{2+}\) quarks, respectively (reviewed in Ref. 371). The initial Ca\(^{2+}\) release activates additional Ca\(^{2+}\) sparks by Ca\(^{2+}\)-induced Ca\(^{2+}\) release from the SR. It is believed today that the signal transmission from the DHPR to the RyR is achieved by mechanical coupling. This is fully compatible with the original hypothesis of Schneider and Chandler (459) that charged components in the sarcolemma and t tubules move in response to depolarization, and this is coupled to a charged component in the SR. That asymmetric charge movement is related to the excitation-contraction coupling could be demonstrated by many studies. For example, it has been shown that in soleus muscle of...
paraplegic rats (after spinal cord transection) the voltage dependence of contraction (twitches and K+ contrac-
tures) and charge movements changed in parallel (109) 
compared with normal animals. Another study shows that 
T_{3}, which shifts the soleus muscles toward fast physi-
ology, also increases the amount of charge movement and 
both the voltage dependence of charge movement and 
tension shifted to more positive potentials (110). Voltage-
dependent depolarization-induced activation is indepen-
dent of a Ca^{2+} inward current (reviewed in Ref. 64).
However, the maintenance of the function of the voltage 
sensor depends on external Ca^{2+}. It seems that Ca^{2+} has 
a stabilizing effect that supports excitation-contraction 
(EC) coupling (reviewed in Refs. 340, 458). In contrast, 
the heart muscle RyR (RyR2) is activated during EC cou-
pling by Ca^{2+} influx through the DHPR, a phenomenon 
referred to as Ca^{2+}-induced Ca^{2+} release (reviewed in 
Ref. 123). Because Ca^{2+} influx through the Ca^{2+} sensor is 
of secondary importance for skeletal muscle physiology, 
this mechanism is not discussed in this article.

In the mouse BC_{3}H_{1} cell line, which serves as a 
model for muscle differentiation, it was found that in the 
proliferative state of the cells the predominant release 
channel was inositol 1,4,5-trisphosphate (IP_{3}) sensitive 
and therefore identified as the endoplasmic reticulum Ca^{2+} channel, whereas after differentiation, the Ca^{2+} mo-
bilization potential was mostly caffeine sensitive, indicative 
of the RyR (SR Ca^{2+} channel) (96b). This suggests that 
differentiation of the BC_{3}H_{1} myoblast phenotype induces the expression of RyR and reduces IP_{3} receptor 
activity, a process which might also take place in muscle 
development in vivo. Investigations on the expression of 
RyR isoforms and IP_{3} receptors during development of 
skeletal muscle or in the specialized adult muscle indicate 
that various combinations of Ca^{2+} release channels could 
contribute to the fine tuning of Ca^{2+} regulation in the 
skeletal muscle (reviewed in Ref. 486).

Because the RyR has a very central position in the 
context of Ca^{2+} handling in muscle physiology and plas-
ticity, it is not surprising that it is also a molecular switch 
that is highly complex and a target of many regulatory 
pathways. We therefore discuss its structure and regula-
tion in some detail and summarize the knowledge on 
putative interacting molecules that could contribute to its 
performance.

The RyR is a homotetramer (see Ref. 324), and 50% of 
all these complexes are located in close proximity to the 
DHPR (131, 140–142, 408). In addition, it has been shown 
that RyR channels are highly clustered square structures 
arranged in regular rows and that the corners of adjacent 
channels contact each other (438). There is ~66% amino 
acid sequence identity among the skeletal, cardiac, and 
brain isoforms (324). Fast and slow skeletal muscle fibers 
contain predominantly one RyR isoform (RyR1), but the 
RyR density is higher in fast fibers (81). Some of the 
biochemical features of the RyR and other molecules 
discussed in this review article are listed in Table 3.

2. Ca^{2+} regulation

Ca^{2+} dependence of the RyR activity is achieved by 
several different mechanisms. The Ca^{2+} release prop-
ties of isolated triad preparations (composed of the ter-


Mg$^{2+}$ cannot permanently inhibit RyR is provided by Hain et al. [178] in that Mg$^{2+}$ can only block the nonphosphorylated state of the RyR which can be phosphorylated by protein kinase A or CaM kinase. The effect of Mg$^{2+}$ is also mediated through phosphatase 2C, which is activated by Mg$^{2+}$ (reviewed in Ref. 469).

An important question concerning intracellular Ca$^{2+}$ storage organelles is how they get refilled after Ca$^{2+}$ is depleted. It is known for many cell types that depletion of intracellular Ca$^{2+}$ stores results in the activation of a store-operated Ca$^{2+}$ channel at the plasma membrane that enables the reloading of internal Ca$^{2+}$ stores. This Ca$^{2+}$ current, the Ca$^{2+}$ release-activated current ($I_{\text{CRAC}}$), may be responsible for long-term Ca$^{2+}$ effects and oscillations. The signals involved in transmitting the information of Ca$^{2+}$ depletion as well as the channel themselves are not well characterized. By single-cell patch-clamp analysis in cultured skeletal muscle cells, Hopf et al. [206] found Ca$^{2+}$ leak channels that were sensitive to two new dihydropyridine compounds, as well as to manganese influx and an inhibitor of tyrosine kinase. Thus the Ca$^{2+}$ leak channel might have an important function for filling the intracellular Ca$^{2+}$ stores in normal contractile activity besides the voltage-dependent Ca$^{2+}$ channel, which also has been shown to mediate the store refilling. However, it was shown that continuous muscle activity in mammalian

| Table 3. Ca$^{2+}$ and calmodulin binding proteins potentially implicated in skeletal muscle function |

<table>
<thead>
<tr>
<th>Protein</th>
<th>Isoforms</th>
<th>$M_r$</th>
<th>Ca$^{2+}$ Binding</th>
<th>CaM Binding</th>
<th>Proposed Function</th>
<th>Selected Reviews</th>
</tr>
</thead>
<tbody>
<tr>
<td>Troponin C</td>
<td>Fast</td>
<td>17,000</td>
<td>+</td>
<td></td>
<td>Myofibril Ca$^{2+}$ sensor protein</td>
<td>152</td>
</tr>
<tr>
<td>Calmodulin</td>
<td>Slow/cardiac</td>
<td>17,000</td>
<td>+</td>
<td></td>
<td>Myofibril Ca$^{2+}$ sensor protein</td>
<td>432</td>
</tr>
<tr>
<td>Calmodulin</td>
<td>S100a</td>
<td>10,000</td>
<td>+</td>
<td></td>
<td>Activation of twitching, possibly other functions</td>
<td>572</td>
</tr>
<tr>
<td>Parvalbumin</td>
<td></td>
<td>12,000</td>
<td>+</td>
<td></td>
<td>Ca$^{2+}$ transport from myofibrils to SR</td>
<td>26</td>
</tr>
<tr>
<td>Myosin light chain 2</td>
<td></td>
<td>17,000</td>
<td>+</td>
<td></td>
<td>Thick filament component</td>
<td>451</td>
</tr>
<tr>
<td>Ryanodine receptor</td>
<td>Skeletal</td>
<td>550,000</td>
<td>+</td>
<td>+</td>
<td>Ca$^{2+}$ release channel of SR</td>
<td>324</td>
</tr>
<tr>
<td>Heart/brain</td>
<td>RyR1</td>
<td>550,000</td>
<td>+</td>
<td>+</td>
<td>Ca$^{2+}$ release channel of SR</td>
<td></td>
</tr>
<tr>
<td>Brain</td>
<td>RyR2</td>
<td>550,000</td>
<td>+</td>
<td>+</td>
<td>Ca$^{2+}$ release channel of SR</td>
<td></td>
</tr>
<tr>
<td>Ca$^{2+}$ pump</td>
<td>Fast adult</td>
<td>110,000</td>
<td>+</td>
<td>+</td>
<td>Ca$^{2+}$ transport into SR</td>
<td>224</td>
</tr>
<tr>
<td>Calpain</td>
<td></td>
<td>80,000</td>
<td>+</td>
<td></td>
<td>Phosphorylation of myosin</td>
<td>502</td>
</tr>
<tr>
<td>Calcineurin A</td>
<td></td>
<td>61,000</td>
<td>+</td>
<td>+</td>
<td>Ca$^{2+}$-dependent phosphatase</td>
<td>171</td>
</tr>
<tr>
<td>Calpastatin</td>
<td>B</td>
<td>17,000</td>
<td>+</td>
<td></td>
<td>Calmodulin-like subunit of calcineurin</td>
<td>560</td>
</tr>
<tr>
<td>Calpastatin</td>
<td>Slow/cardiac</td>
<td>45,000</td>
<td>+</td>
<td></td>
<td>Ca$^{2+}$ storage protein of the SR</td>
<td></td>
</tr>
<tr>
<td>Calpastatin</td>
<td></td>
<td>60,000</td>
<td>+</td>
<td></td>
<td>Ca$^{2+}$ storage protein of the SR</td>
<td></td>
</tr>
<tr>
<td>Annexin VI</td>
<td>Slow</td>
<td>68,000</td>
<td>+</td>
<td></td>
<td>Ca$^{2+}$-dependent phospholipid binding</td>
<td>113</td>
</tr>
<tr>
<td>Sorcin</td>
<td></td>
<td>22,000</td>
<td>+</td>
<td></td>
<td>Transsarcolemmal transport?</td>
<td>345</td>
</tr>
<tr>
<td>NO synthase (nNOS)</td>
<td></td>
<td>160,000</td>
<td>+</td>
<td></td>
<td>Possibly involved in relaxation</td>
<td>42</td>
</tr>
<tr>
<td>a-Actinin</td>
<td></td>
<td>100,000</td>
<td>+</td>
<td></td>
<td>Thin filament component</td>
<td>373</td>
</tr>
<tr>
<td>Dystrophin</td>
<td>Slow/cardiac</td>
<td>500,000</td>
<td>+</td>
<td></td>
<td>Connects thin filaments with sarcotome</td>
<td>6</td>
</tr>
</tbody>
</table>

NO, nitric oxide; CaM, calmodulin; RyR, ryanodine receptor; SERCA, sarco(endo)plasmic reticulum Ca$^{2+}$-ATPase; SR, sarcoplasmic reticulum.
skeletal muscle causes a dramatic increase of total muscle Ca$^{2+}$ (122). However, Ca$^{2+}$ concentrations in store organelles were not measured directly in these studies.

Taken together, the importance of the RyR for Ca$^{2+}$-regulated muscle function can hardly be overemphasized. This large protein complex provides on the one hand the entry site for activating signals coming through surface membrane depolarization and DHPR activation. On the other hand, it releases Ca$^{2+}$ from the SR, a process which is tightly controlled by the concentration of Ca$^{2+}$ in the SR as well as by many other factors playing regulatory roles with mostly unknown molecular mechanisms.

3. Proteins that interact with RyR

In skeletal muscle, direct interaction of the DHPR in the t tubules of the plasma membrane with the RyR in the SR is believed to be responsible for EC coupling via a structural change in the DHPR that induces a structural alteration in the RyR, which finally triggers the opening of Ca$^{2+}$ release channels (426) (see Fig. 4). Biochemical evidence for a link between the two receptors has been reported by Marty et al. (327). RyR interact with a variety of accessory proteins believed to modulate the activity of these Ca$^{2+}$ channels (reviewed in Ref. 317). The following proteins have been shown to bind directly to the RyR or affect the gating properties of the RyR: gyceraldehyde-3-phosphate dehydrogenase (41), aldolase (41), annexin VI (97), 170-kDa low-density lipoprotein binding protein (80), S100 protein (124, 325), CaM, 60-kDa CaM-dependent protein kinase (82), calsequestrin (365, 560), FK506 binding protein (233), triadin (173), and junctin (238). Whether some of these proteins use common docking sites on the RyR is presently not known. Because this review focuses on Ca$^{2+}$-related issues, we discuss in more detail calsequestrin and CaM. They are both potentially involved in Ca$^{2+}$-governed RyR function and regulation. In addition, new literature on FK506 binding protein is reviewed since this protein seems to have a major function for RyR regulation.

4. Calsequestrin

Calsequestrin is the main Ca$^{2+}$-binding protein of the SR, with high capacity and low affinity for Ca$^{2+}$. The biochemical features of this protein are discussed in section ivD (see also Table 3). Calsequestrin contains no transmembrane segments and is therefore believed to be located within the lumen of the SR (132, 387). A region of the protein (amino acid 86–191) was shown to bind to the functional face membrane of the SR (71). Experiments with ryanodine receptor Ca$^{2+}$ release agonists, such as polylysine or caffeine, suggest that the RyR, when activated by such agents, induces a release of Ca$^{2+}$ from calsequestrin (219, 464). Experiments using fluorescent probes that were bound to SR proteins point in the same direction. In the presence of calsequestrin, the fluorescence intensity of the probe increased with luminal Ca$^{2+}$ but not in its absence (220). Therefore, it was concluded that the Ca$^{2+}$-dependent conformational change of calsequestrin causes a change in the shape of SR membrane proteins, including the RyR. A recent study shows that calsequestrin controls the RyR channel in a phosphorylation state-dependent fashion. Calsequestrin, exclusively when phosphorylated, enhanced the open probability of the RyR fivefold and increased the open time twofold (509). It is possible that calsequestrin interacts with the 95-kDa protein triadin, a protein thought to bind to both the RyR and the DHPR (63). Because triadin contains a region of basic amino acids in the luminal domain, it was suggested that it could interact with the acidic protein calsequestrin and provide a functional connection to the RyR (355). Recently, it was found that the luminal domain of triadin interacts with calsequestrin in a Ca$^{2+}$-dependent manner and therefore triadin might anchor calsequestrin to the junctional region of the SR and might thus be important for functional coupling in the Ca$^{2+}$ cycle (172). Purified triadin inhibits $[^{3}H]$ryanodine binding to the solubilized heavy fraction of the SR of rabbit skeletal muscle and reduces the opening of the RyR (380). The same study shows that calsequestrin potentiates the Ca$^{2+}$-dependent $[^{3}H]$ryanodine binding and that this effect was reduced by triadin. That triadin functionally interacts with
the skeletal RyR was also shown by antibodies directed against the COOH-terminal part of triadin, which induces a decrease in the rate of Ca$^{2+}$ release from SR vesicles as well as a decrease of the open probability of the RyR Ca$^{2+}$ channel incorporated in lipid bilayers (167). Junctin, another calsequestrin binding protein of 26 kDa with sequence homology to triadin, was found abundantly in junctional membranes and could therefore as well serve to bring calsequestrin in proximity to the RyR (559). In a recent study, the close proximity of calsequestrin and the RyR in fast- and slow-twitch rabbit skeletal muscle was demonstrated by immunoblot analysis of chemically cross-linked membrane vesicles enriched in triad junctions (365).

5. CaM

CaM binds to the RyR and affects its function in a complex positive and negative way (357, 465). CaM is a ubiquitous intracellular Ca$^{2+}$ receptor containing typical EF-hand structural elements that bind Ca$^{2+}$ in a specific way (see Fig. 9 and Ref. 311). The term EF-hand refers to the two COOH-terminal α-helical sequence stretches in PV which are oriented in a perpendicular way and connected by a loop that contains amino acid residues serving as Ca$^{2+}$ ligands. Proteins containing such structural elements are named EF-hand proteins (272). Generally, at submicromolar Ca$^{2+}$ concentrations (10$^{-7}$ to 10$^{-9}$ M), CaM activates the channel by increasing the open probability in a dose-dependent fashion, and at high free Ca$^{2+}$ (10 μM), CaM (0.1–1 μM) inhibits channel activity (53). It has been shown that CaM inhibits Ca$^{2+}$ release from skeletal SR vesicles by a factor of 2–3 (337). Half-maximal inhibition was found between 0.1 and 0.2 μM and maximal inhibition at 1–5 μM CaM. The bell-shaped Ca$^{2+}$ dependence of Ca$^{2+}$ release (see Fig. 5) between 0.1 and 100 μM was not shifted by CaM. Only its amplitude was altered. In the absence of ATP, CaM decreased the mean open time with known catalytic or regulatory functions in the skeletal muscle are phosphorylase kinase (188), glycogen synthase kinase (552), CaM kinase II (250), calcineurin (171) (see also sect. ivE), NO synthase (42), and dystrophin (6). For biochemical features, see Table 3. A more complete list of CaM targets is presented in Reference 432. Recently, Pyk2, a stress-related kinase that signals through mitogen-activated protein kinase and promotes apoptosis, has been proposed to be activated by CaM (90). Another newly discovered group of enzymes shown to be Ca$^{2+}$/CaM dependent are DAPK, serine/threonine kinases, involved in apoptotic Ca$^{2+}$ signaling and shown to be present in the skeletal muscle (70, 245). It is not clear whether all these mentioned pathways are important for gene regulation in skeletal muscle, but all the necessary components have been shown to exist in muscle cells.

6. FK506 binding protein

The tetrameric RyR channel binds four molecules of the FK506 binding protein (233), which has a stabilizing effect on the RyR and coordinates its activity (43). In a recent article it has been postulated that FK506 binding protein also coordinates the opening of several adjacent channels to release Ca$^{2+}$ in a simultaneous way (328). Such a coupled gating would allow the regulation of channels that are not associated with voltage-dependent channels.

7. Summary

Taken together, the association of the RyR with the Ca$^{2+}$-binding proteins calsequestrin and CaM provide many possibilities for regulating the important Ca$^{2+}$ channel activity in the cell. In addition to direct binding of Ca$^{2+}$-regulated cellular components, Ca$^{2+}$ can also affect the RyR in an indirect fashion through Ca$^{2+}$/CaM-dependent phosphorylation or dephosphorylation. Considering different RyR and calsequestrin isoforms and the regulation of the expression level, the RyR Ca$^{2+}$ channel is
clearly an important component in processes that govern muscle plasticity.

B. Malignant Hyperthermia, a Disease of Calcium Release

The RyR that has been discussed in the previous section plays a crucial role in malignant hyperthermia (MH). MH is known in humans (95, 319) and pigs; it is caused by a pathophysiological response of skeletal muscle to some anesthetics and muscle relaxants. An MH crisis is life threatening and one of the main causes of death during general anesthesia in humans. The occurrence has been estimated to be between 1:12,000 and 1:40,000 general anesthetics (351). An attack starts with muscle hypermetabolism, contractures, and a following dramatic rise in body temperature [up to 1°C per 5 min and up to 43°C (hyperthermia) (168)]. The susceptibility to develop MH (MHS) during anesthesia is genetic and is transmitted as an autosomal dominant trait. MHS can be tested by exposure of biopsied muscle bundles to trigger substances, such as caffeine, halothane, succinylcholine, ryanodine (116, 283, 513a), or 4-chloro-m-cresol (197). When muscle bundles of individuals with MHS are exposed to one of these drugs, the bundles show faster force production or respond at lower drug concentrations compared with controls. This has led to the design of standardized in vitro contracture tests (283, 513b), which are widely used for testing MHS.

Although MHS can be life threatening during general anesthesia, the affected individuals appear to be without symptoms during normal life. The disposition is genetically heterogeneous, but the common underlying effect leading to a MH crisis is an excess of Ca\(^{2+}\) in the sarcoplasm (Fig. 6; Ref. 351). Secondary changes are the clinically observed muscle contractures, a high rate of ATP consumption and synthesis, and the increase in muscle temperature and body temperature. If the process cannot be terminated by pharmacological means, i.e., the application of dantrolene (540), an inhibitor of the SR Ca\(^{2+}\) release, muscle tissue gets severely damaged and very soon secondary effects including heart failure, renal failure, and neurological complications can occur (319).

Porcine MH develops principally in an identical way and can be provoked with the same trigger substances: halothane, succinylcholine, or 4-chloro-m-cresol (218). However, pigs that are homozygous for the genetic disposition also respond to stress with muscle stiffness, hypermetabolism, and high temperature. Animal transportation in a hot environment can lead to stress-induced death (porcine stress syndrome) (319, 417). Another difference between human and porcine MHS is the tendency of faster growth and a faster increase in muscle mass of MHS pigs compared with controls. This property, which was desired and selected by the breeders, points to the side effect of hypertrophy of porcine MHS muscle. The mechanism of hypertrophy is not yet clarified, but as in mdx muscle (see sect. v), a sustained increase of cytoplasmic Ca\(^{2+}\) and subsequent calcineurin-dependent signal transduction into the nucleus (366, 468) could underlie this effect (see Figs. 3, 13, and 14).

1. MH caused by mutations in the RyR1 gene

The disposition for MH is genetically heterogeneous in humans, and the underlying molecular defects are known only in a fraction of the affected families. In ~50% of the affected patients, point mutations in the RyR1 gene, coding for the major Ca\(^{2+}\) release channel of skeletal muscle, have been made responsible for MHS. All the known 25 different mutations (39, 315, 323) are point mutations causing amino acid exchanges in the RyR1 (Fig. 6).
(Fig. 4). The first detected mutation, R614C in humans (154), is homologous to the mutation causing MHS in pigs, R615C (211). Ryanodine receptors with the R615C mutation show an increased [3H]ryanodine binding in the presence of Ca\(^{2+}\) (350) and have an increased open probability compared with control when incorporated into bilayers (127). The latter effect has recently been attributed to a lower sensitivity of R615C mutated RyR to Mg\(^{2+}\). The inhibition of the RyR by Mg\(^{2+}\) is probably physiologically important to adjust a normal level of activity of the RyR. Thus the loss of Mg\(^{2+}\) sensitivity could lead to overactivity of the release channels (290, 291). Furthermore, SR vesicles with the mutated RyR1, prepared from pig or human muscle, show a higher affinity for ryanodine and increased sensitivity for Ca\(^{2+}\) (349). The RyR1 with the R615C mutation was shown to be more sensitive to activating CaM concentrations (378) and muscle fibers, and myotubes from pigs with MHS have a lower threshold for contraction upon electrical stimulation and exposure to extracellular K\(^+\) (147). Recently, it was shown that the G2434R mutation of the human RyR1 causes similar changes of physiological and pharmacological properties as the R614C mutation. The G2434R mutation also caused a higher sensitivity of the RyR to activating Ca\(^{2+}\) concentrations, when tested in membrane preparations with a ryanodine binding assay. In addition, the sensitivity of the mutated channel to caffeine and 4-chloro-m-cresol was increased (420), and inhibition by high Ca\(^{2+}\) concentrations and CaM were reduced. All known MHS causing mutations of the RyR1 are localized in the proposed cytoplasmic part of the release channel, the domain responsible for the coupling to the DHP receptor (Fig. 4). The mutations seem to confer the RyR into a hypersensitive state leading to excessive Ca\(^{2+}\) release after direct binding of trigger substances or by activation via depolarizing agents and after DHPR activation.

Although excessive Ca\(^{2+}\) release into the sarcomplasm is common in MH and although the mutated RyR may show similar physiological properties (420), there is evidence for differences in phenotypes in the absence of triggering agents. Some mutations appear to have no effect in the absence of drugs, i.e., they probably do not cause changes in cytoplasmic Ca\(^{2+}\) (compared with controls) during normal muscle activity. Some mutations, as the R615C in pigs, lead to hypertrophy, probably by increased cytoplasmic Ca\(^{2+}\) at rest or during normal muscle activity. A third category of MH-causing mutations (39, 315, 402) is at the same time responsible for central core disease (CCD). This muscle disorder is characterized by central cores of debris in muscle fibers. For this the best explanation is a marked Ca\(^{2+}\) release through the mutated RyR at rest (315) and following Ca\(^{2+}\)-dependent degradation of myofibrils and mitochondria, which then form the central cores.

2. Other genetic defects related to MH

To complete the discussion on MH, a short overview on other MH candidate loci, unrelated to the RyR locus, is given. As mentioned above, RyR1 gene mutations (chromosome 19q13.1) account only for a part of the MHS cases. This first gene locus for MHS was named MHS1. On the basis of genetic linkage studies, there is evidence for at least five other MHS loci called MHS2 (chromosome 17q11.2-q24, Ref. 300), MHS3 (chromosome 7q, Ref. 222), MHS4 (chromosome 3q13.3, Ref. 503), MHS5 (chromosome 1q31, Ref. 356), and MHS6 (chromosome 5p, Ref. 431). Candidate genes exist for some of the loci (Table 2). The MHS5 locus contains a chromosomal segment that includes the gene for the αδ-subunit of the skeletal muscle L-type Ca\(^{2+}\) channel, but a mutation has not been found in the gene. In any case, the linkage data added evidence for a considerable heterogeneity of MHS. Recently, MHS5 was confirmed as an independent MHS locus after a mutation in the CALM1A3 gene (356), coding for the α-subunit of the muscular L-type calcium channel had been found.

3. Summary

In summary, MH is a disorder of Ca\(^{2+}\) release of skeletal muscle with the phenotype of increased sensitivity of muscle to certain trigger substances used during anesthesia. The finding that MHS can be caused by mutations in the RyR1 and the muscular L-type Ca\(^{2+}\) channel points to the whole complex of associated proteins which form the t-tubule/SR connection (Figs. 4 and 6). Structural integrity and function of this complex seem to be highly important for the normal process of muscle activation. To clarify the genetic and physiological basis of the MHS loci 2, 3, 4, and 6 will lead to great progress for the understanding of the skeletal muscle Ca\(^{2+}\) control. The fact that the myoplasmic Ca\(^{2+}\) level can get out of control as a consequence of changes of the RyR function underlines the importance of this component of the calcium cycle.

C. The Calcium Switch at the Myofibrils: the Troponin Complex and Calcium Control at the Myofibrils

1. Structure and function

After its release from the SR, Ca\(^{2+}\) binds in a fast reaction to one of the troponin subunits (TnC) which forms the regulatory complex with tropomyosin on the thin filament (112, 152, 411) (Fig. 7). This event is followed by a transient tension development at the contractile apparatus leading to muscle contraction.

As reflected in Ca\(^{2+}\) dependence curves, the Ca\(^{2+}\) concentrations leading to half-maximal tension development are markedly different in type I, IIA, and IIB muscle fibers. TnC, one prominent factor in the Ca\(^{2+}\) cycle, influ-
nces the sensitivity differentially by displaying two different isoforms. Fiber type I contains TnCs (slow, identical with the heart form) and both type IIA and IIB contain TnCf (fast). The fact that TnCf contains two, and TnCs only one, regulatory Ca$^{2+}$-binding site does not fully explain the physiological difference (for further discussion, see Ref. 453). It has to be mentioned that although TnC binds Ca$^{2+}$ directly, it is not the only factor responsible for the myofibrillar Ca$^{2+}$ sensitivity. The other troponin subunit isoform as well as the tropomyosin, myosin, and myosin binding proteins affect the Ca$^{2+}$ sensitivity too. Ca$^{2+}$-dependent force generation is depressed by low pH. The depression is highest in cardiac muscle, intermediate in fast muscle, and lowest in slow muscle. A recent study, where different isoforms of the troponin complex were exchanged in skinned cardiac muscle fibers, shows that

TnC is the determinant of the differential pH sensitivity of the fast and skeletal muscle, whereas it is TnI in the slow muscle (358).

TnCf binds two Ca$^{2+}$ in a fast reaction and with moderate affinity ($5 \times 10^6$ M$^{-1}$) in the NH$_2$-terminal part of the molecule, and two further Ca$^{2+}$ bind with slow kinetics and with high affinity ($5 \times 10^8$ M$^{-1}$) in the COOH-terminal part (430) (for kinetics of Ca$^{2+}$ exchange, see also Fig. 8).

As a consequence of Ca$^{2+}$ binding to TnC, a movement of TnI releases the inhibitory effect of the troponin complex and allows thin and thick filaments to interact as shown by resonance energy transfer and cross-linking experiments (511).

TnC belongs to the superfamily of EF-hand Ca$^{2+}$-binding proteins, of which PV (see sect. IV:A) was the first member to be analyzed at the three-dimensional level (for
a review, see Ref. 272). PV contains the prototypic high-affinity Ca\(^{2+}\) binding sites that were later found in all other members of this superfamily. The Ca\(^{2+}\)-binding sites of the EF-hand structure (see Fig. 9) provide the coordinating ligands for Ca\(^{2+}\) at the vertices of a pentagonal bipyramid. TnC contains 8 \(\alpha\)-helices designated A-H and a short NH\(_2\)-terminal \(\alpha\)-helix.

In a study where all four Ca\(^{2+}\) sites of chicken skeletal muscle TnC were individually mutated, it could be demonstrated that the NH\(_2\)-terminal sites are needed for regulation and that both COOH-terminal sites are needed for binding of TnC to the thin filament (483). This is in contrast to results obtained for the cardiac TnC, where it seems that metal binding to one site of the COOH-terminal part is sufficient (369) for both functions.

2. Conformational change upon Ca\(^{2+}\) binding

The molecular nature of the conformational change in TnC, which is Ca\(^{2+}\) dependent and needed for triggering muscle contraction, is not entirely understood. Based on the structural model by Herzberg et al. (198) and a series of mutagenesis experiments, Gergely et al. (152) proposed that the conformational shift consists of a Ca\(^{2+}\)-induced angular movement of one pair of helical segments relative to another pair of helices in the NH\(_2\)-terminal domain. By a joint movement of helices B and C away from helices A and D, the structure of the protein is changed in a Ca\(^{2+}\)-dependent fashion. This structural change exposes a hydrophobic patch that could be important for TnI binding. Another site of similar structure is formed by the helical segments in the COOH-terminal domain. This view is supported by the fact that introduction of a disulfide bridge into the NH\(_2\)-terminal domain blocks the conformational change and consequently also the Ca\(^{2+}\) regulatory activity (152). Reduced TnC used as a

![Diagram of calcium ions and tropomyosin](image)

**FIG. 8.** Kinetics of metal exchanges with troponin C and parvalbumin. Approximate values are according to Reference 405 (see also Ref. 390 for further literature). Kinetics of Ca\(^{2+}\) binding to troponin C (regulatory sites) and parvalbumin dictate the order of the flow of released Ca\(^{2+}\) to first troponin C followed by binding to parvalbumin in the fast skeletal muscle. For simplicity, kinetics of the troponin C high-affinity sites are neglected. They are in the range of the constants for parvalbumin. The oval symbol is used for troponin C, and the square is used for parvalbumin. Open symbols indicate the metal free state, and solid symbols indicate the metal loaded state.

![Structures of parvalbumin and CaM in stereo view](image)

**FIG. 9.** Structures of parvalbumin and CaM in stereo view. Comparison of the structures of \(\alpha\)-parvalbumin and CaM (COOH-terminal domain) both in the presence of Ca\(^{2+}\). The main structural units are colored in blue, yellow, and red with increasing freedom of local mobility. For details see Reference 17. (Figure kindly provided by Dr. Balledelon, Montpellier, VT.)
3. Interaction with other troponin subunits

How the two lobes of TnC interact with TnI to loosen its association with actin is beginning to be unraveled. TnC makes multiple Ca\(^{2+}\)-dependent and Ca\(^{2+}\)-independent interactions with TnI and TnT. The so-called inhibitory segment in the middle of TnI (residues 96–116) has been shown to interact with the C-helix in the NH\(_2\)-terminal domain of TnC (263, 299) and also with regions in the COOH-terminal part of TnC. It seems that the interactions at both sites are necessary for full regulatory activity of TnC. The interaction with the COOH-terminal site is necessary for stabilization of the complex, and the one of the NH\(_2\)-terminal domain is directly coupled to the release of inhibition by TnI. A recent NMR study using TnI peptides and chicken skeletal TnC shows that residues 97–136 of TnI are involved in binding to the two lobes of TnC under Ca\(^{2+}\)-saturating conditions and that the interaction with the regulatory domain of TnC is complex (392). It seems that particularly acidic residues (E53, E54, E60, E61 and E85, D86) are important for the complex protein-protein interaction among TnI and TnC (264, 265). TnI seems to associate with TnC in an antiparallel fashion. The COOH-terminal part of TnC interacts with the NH\(_2\)-terminal part of TnI and vice versa (125, 262). In a binary complex, both proteins are in an extended conformation, and TnI seems to wrap around TnC (383). In the absence of Ca\(^{2+}\), the inhibitory region in TnI binds to actin and inhibits ATPase activity. By binding to TnC in the presence of Ca\(^{2+}\), this domain is moved away from actin, and inhibition is relieved. This is followed by a displacement of tropomyosin.

Whereas a multiplicity of fast and slow forms of TnT isoforms exists, TnC and TnI each occur only in a slow and a fast form. The expression of all troponin forms in skeletal muscle is under neuronal control. During muscle transformation, e.g., from fast to slow, isoforms of all three troponin forms change. Fast and slow isoforms of all three subunits may coexist in transforming muscle fibers, indicating an enormous plasticity of the troponin switch system (182).

The interaction of actin with myosin affects the troponin system in a reverse fashion. Ca\(^{2+}\) binding to TnC is enhanced by cross-bridge attachment. By this, the Ca\(^{2+}\) signal is amplified. Ca\(^{2+}\) binds first to troponin, activating myosin binding to the thin filament. This leads to additional cross-bridge attachments through enhanced Ca\(^{2+}\) binding or by changing the thin filament structure directly (37).

4. Summary

In summary, the Ca\(^{2+}\) transiently released from the SR through the RyR activates muscle contraction utilizing TnC as a direct target. Ca\(^{2+}\) binding to TnC changes its structure and influences other troponin subunits, finally leading to activation of the myosin ATPase. This cascade of events finally leads to muscle contraction. Similar to the switch at the RyR also this event can be fine-tuned to the need of an individual cell type, muscle, or developmental stage if isoform diversity of the troponin subunits are considered.

A. Parvalbumin as a Relaxation Factor in Fast-Twitch Muscle Fibers

1. Structure and proposed function

Skeletal muscle relaxation after a single twitch or a tetanic contraction is initiated by a fall in sarcoplasmic Ca\(^{2+}\) concentration. The following three consecutive steps of Ca\(^{2+}\) removal largely dictate the efficiency of the relaxation process: dissociation of Ca\(^{2+}\) from TnC, translocation near the site of entry into the SR, and uptake into the SR by the Ca\(^{2+}\)-Mg\(^{2+}\)-ATPase (the “Ca\(^{2+}\) pump”). It is not known which of these mechanisms is rate limiting in slow-relaxing muscle fibers. Interspecies comparison (191) as well as force measurements of single slow- and fast-twitch muscle fibers (210) showed a positive correlation between PV content and relaxation rate, indicating that PV could facilitate Ca\(^{2+}\) translocation within the sarcoplasm and therefore be a limiting factor for relaxation in fast-twitch muscles. It is generally agreed that PV, which has relatively slow on/off rates for Ca\(^{2+}\) (see Fig. 7), would not compete for Ca\(^{2+}\) with TnC, but would rather bind Ca\(^{2+}\) after the triggering of muscle contraction. PV can be detected in fast-contracting/relaxing muscle fibers of rodents starting 4–6 days after birth. The time period of the maximal increase in PV coincides with the differentiation of the fast-twitch muscle function, indicating that PV is involved in fast-twitch muscle function. Therefore and for reasons mentioned later, it was proposed that this protein could act in the process of muscle relaxation by facilitating Ca\(^{2+}\) transport from the myofibrils into the SR (151, 156, 175, 392). However, no direct evidence for an involvement of PV in the relaxation process has been obtained until very recently when mice with deleted PV genes were analyzed (462).

PV is a high-affinity Ca\(^{2+}\)-binding protein found at high concentration in fast-contracting/relaxing skeletal muscle fibers of vertebrates (reviewed in Refs. 26, 27, 390). In rat and mouse, the type IIB fibers show the strongest immunoreactivity for PV with different degrees
of intensity (Fig. 2). The majority of type IIA fibers (60–70%) exhibit moderate (also graded) staining intensity. The remaining type IIA and the type I fibers lack PV (65, 144, 190, 363). The different muscle fibers of the rabbit exhibit a very similar distribution of PV (292, 456). In human muscles, PV is detectable exclusively in intrafusal fibers (137).

This protein has a molecular mass of 12 kDa and binds two \( \text{Ca}^{2+} \) with high affinity. Its structure and for comparison that of calmodulin (only COOH-terminal part) are shown in Figure 9. Interestingly, PV, which binds both \( \text{Mg}^{2+} \) and \( \text{Ca}^{2+} \), has a much more rigid structure compared with CaM, which exclusively binds \( \text{Ca}^{2+} \) but with lower affinity as PV (17). For carp PV, Robertson et al. (430) calculated dissociation rates in the range of 10 and \( 1 \text{ s}^{-1} \) for \( \text{Mg}^{2+} \) and \( \text{Ca}^{2+} \), respectively (Fig. 7). Because half-relaxation time is generally much below 100 ms after a single twitch, it has to be assumed that the dissociation rates of metals from PV are quite different in vivo compared with values obtained in vitro, allowing a rapid exchange of \( \text{Ca}^{2+} \) which is important for muscle relaxation, because the more recently measured \( K_{\text{Ca}} \) affinity constants (\( K_{\text{Ca}} \)) for PV are generally severalfold lower than estimated earlier (especially for \( K_{\text{Ca}} \) values obtained from PV of mammals); PV may also play an important role in muscle relaxation after a single twitch.

2. Functional experiments

Cross-reinnervation of slow- and fast-twitch muscles which changes physiological and biochemical properties of the muscles leads to an alteration of the PV content. In the cross-reinnervated fast-twitch muscle, the PV content is decreased whereas in the cross-reinnervated slow muscle PV amounts increase (360, 362) (see sect. tC). PV changes are among the first alterations observed during transformation and are followed by changes of the contractile apparatus. Electrical stimulation of fast-twitch muscle leading to a slow phenotype decreases the PV content (293, 397). PV concentrations in fast fibers of small mammals are higher than in fast fibers of bigger animals (191). The latter fibers are known to contract and relax slower than the corresponding ones in small animals. All these studies indicate that PV is associated with fast-twitch muscle function and predict a functional role of PV in the relaxation process. However, based on metal binding kinetic properties of PV in vitro, this hypothesis has been challenged (236, 405, 430).

To test whether PV could act as a relaxing factor, direct gene transfer (1, 535, 551) was applied in normal and regenerating rat soleus muscles that do not synthesize detectable amounts of PV (361). Two weeks after in vivo transfection with PV cDNA under the control of various viral promoters, considerable levels of PV mRNA and protein were detected in uninjured and even higher amounts in regenerating muscles. Half-relaxation time was significantly shorter in transfected than in nontransfected muscles or in muscles transfected with nonfunctional cDNA constructs, whereas time to peak and twitch-to-tetanus ratios as well as force production remained unchanged (Fig. 10). The inverse correlation between half-relaxation time and PV concentration in the transfected muscles in a dose-dependent fashion directly demonstrated for the first time the physiological function of PV as a relaxing factor in mammalian fast-twitch skeletal muscles (361).

Recently, the function of PV in fast-contracting/relaxing muscles could also be directly demonstrated in mice lacking PV due to gene knock out (462). The decrease of \( \text{Ca}^{2+} \) concentration seen after a 20-ms stimulation of the isolated extensor digitorum longus was slower (33% lower rate constant of \( \text{Ca}^{2+} \) decay) in knockout mice compared with wild-type animals. This led to an increase of the half-relaxation time. In addition, because of a higher \( \text{Ca}^{2+} \) concentration in the muscle, the force generated during a single twitch was 40% higher in PV(−/−) mice compared with PV (+/−) and PV (+/+ ) mice. In contrast to the results obtained by direct gene transfer (361) the knockout experiment shows that the presence of PV may also shorten the time needed to obtain peak twitch tension. Comparing these two experiments, one has to consider that in the direct gene transfer experiment a slow muscle (soleus) with ectopic PV expression was investigated, whereas in the knock-out experiment a fast muscle (extensor digitorum longus) with completely abolished PV expression was analyzed. These results clearly demonstrate that PV plays a critical role in fast and phasic muscle contraction.
3. Summary

In conclusion, the majority of experimental data points to the function of PV as a relaxing factor in the specialized fast-twitch muscle. The millimolar PV concentration in these muscles seems to be needed mostly for enabling relaxation after prolonged contraction. Proof of this hypothesis is now available from direct gene transfer experiments as well as from analysis of PV knockout mice.

B. Calcium Uptake by SERCA

1. Structure and function

After Ca\(^{2+}\) has travelled through the RyR Ca\(^{2+}\) release channel and activated the TnC muscle contraction switch system, it has to be pumped back into the SR by the ATP-dependent Ca\(^{2+}\) pumps, which represents the third member of the crucial Ca\(^{2+}\) cycle. The dissociation constant for Ca\(^{2+}\) is \(0.5 \text{ mM}\), and a maximal velocity of 6.4 and 2.4 \(\mu\text{mol-g fiber protein}^{-1}\text{-min}^{-1}\) in fast and slow single fibers, respectively (441), has been measured.

SERCA belong to the cation-transport ATPases occurring most likely in a tetrameric form (for more biochemical features, see Table 3). It is believed that a single polypeptide spans the membrane 10 times and that the NH\(_2\) terminal as well as the COOH terminal and the major part of the protein are located in the cytosol (reviewed in Ref. 225) (see Fig. 11). A variety of analyses have revealed that SERCA are structured as a globular lobe protruding into the cytosol and connected with the membrane through a stalk. It seems that the luminal part of the protein is only minor. Aspartic acid at position 351 in the large extramembraneous domain was identified as the catalytic site undergoing phosphorylation as an intermediate step of the catalytic cycle.

The cation-binding site is located in a membrane domain at some distance (\(\sim 5\) nm) from the catalytic site. Binding of Ca\(^{2+}\) occurs in a protein crevice where one Ca\(^{2+}\) is bound deeply and its outward dissociation can be blocked by a second Ca\(^{2+}\) bound less deeply in the same crevice. It was proposed that the four amphiphilic helices that contain the six residues involved in Ca\(^{2+}\) binding as identified by site-directed mutagenesis are clustered in

![Diagram of Ca\(^{2+}\) pump structure](image-url)

**Fig. 11.** The Ca\(^{2+}\) pump. Structure of the Ca\(^{2+}\)-ATPase is according to Bigelow and Inesi (29). A: overall 3-dimensional structure of the Ca\(^{2+}\)-ATPase. A large segment (50% of the total protein) protrudes on the cytosolic side of the membrane with a narrow stalk, connected to a large head. Ten helical segments cross the membrane. Very little of the protein extends into the luminal side of the membrane. B: diagram of the distribution of the protein sequence in the cytosolic, transmembrane, and luminal space. The cytosolic head structure is composed of two major segments. The larger, residues 319–763, contains the catalytic site Asp-351, which is phosphorylated during the catalytic turnover, and the ATP binding site. The smaller cytosolic loop, residues 107–261, is likely to participate also in the folded structure. The six residues essential for Ca\(^{2+}\) binding are shown within the membrane in helices 4, 5, 6, and 8. The splice mutation is marked by the triangles. The diagram is essentially derived from the analyses of MacLennan et al. (318) and Clarke et al. (69). [Modified from Inesi and collaborators (224, 225).]
such a way that optimal binding of cations is provided and a channel can be formed (see Fig. 11). Interestingly, one of the residues involved in Ca\(^{2+}\) binding (Glu-309) is in transmembrane helix 4 which continues into the cytosol into a domain containing Asp-351. The entire sequence from position 309 to 351 could adopt a helical configuration. Therefore, displacement or rotation of this helix caused by phosphorylation could directly affect Ca\(^{2+}\) binding. This sequence is highly conserved among Ca\(^{2+}\) and other transport ATPases, indicating an important role in the functional linkage between ATP hydrolysis and cation pumping.

It has been proposed that the cooperative Ca\(^{2+}\) binding in the ATPase has similar features as found in the crystal structure of Ca\(^{2+}\)-binding protein thermolysin. Interestingly, in the duplex binding site of thermolysin, three acidic side chains are shared by both Ca\(^{2+}\). On the basis of the knowledge of the critical residues for Ca\(^{2+}\) binding in the ATPase, it is reasonable to speculate that this enzyme adopts a very similar Ca\(^{2+}\) coordination to that found in thermolysin. A large amount of experimental data supports the idea that a single conformational change of the pump from state E1 to E2 may explain how Ca\(^{2+}\) can be pumped through the membrane by the Ca\(^{2+}\)-ATPase: E1 and E2 conformational stages would display high and low cation binding affinities with different vectorial orientations, respectively. The equilibrium between the two states would be controlled by ligand binding and ATP consumption (for review, see Ref. 224). On the basis of kinetic studies it was possible to construct energy diagrams for the catalytic and transport cycle. One mole of enzyme binds 2 mol Ca\(^{2+}\) cooperatively. Active transport is dependent on the functional linkage between the phosphorylation and the Ca\(^{2+}\)-binding domain. Interaction of Ca\(^{2+}\) with the pump occurs in a stepwise fashion, indicating that most likely several conformational changes have to be executed to reorient the enzyme with respect to the membrane. Conformational change is needed for the cooperative binding of two Ca\(^{2+}\), occlusion of the bound Ca\(^{2+}\), and progress through a channel formed by transmembrane helices.

2. Isoform expression

SERCA are encoded by three different genes which by alternative splicing are able to encode five different isoforms. Expression of these proteins is tissue specific and developmentally regulated (556). SERCA1 is found exclusively in the skeletal muscle, exhibits a fast-twitch specific expression, and produces an adult isoform (SERCA1a) and a neonatal (SERCA1b) isoform. On the other hand, SERCA2 is expressed in all tissues. SERCA2a is a muscle-specific protein (heart, slow-twitch skeletal muscle, and smooth muscle) appearing very early in development, whereas SERCA2b is found in nonmuscle tissues and in smooth muscle cells. In the cell line BC\(_3\)H1 it was shown that development of the myogenic phenotype involves the activation of expression of SERCA2 and also induced an isoform switch from isoform SERCA2b to SERCA2a. Myoblasts expressing SERCA2a showed a decreased sensitivity of Ca\(^{2+}\) uptake to the Ca\(^{2+}\) pump inhibitor thapsigargin, suggesting functional differences that could have a profound effect on Ca\(^{2+}\) handling and muscle plasticity (96a). During regeneration from norepinephrine-induced skeletal muscle necrosis of rat soleus muscle, SERCA expression was shown to be regulated in an isoform-specific fashion (566). In SERCA2a, the COOH-terminal stretch of SERCA2b of 49 amino acids is replaced by 4 amino acids leading to functional differences (316, 533). Isoform switching requires activation of a specific splice process at the 3’-end of the primary gene transcript. This seems to be a specifically regulated process and not due to generally altered splicing activity or decrease of polyadenylation efficiency at the upstream polyadenylation site and is only active in differentiated muscle cells (528). SERCA3 is expressed at high levels in platelets, lymphoid cells, and some endothelial cells (33, 555). By in situ hybridization, Wu et al. (554) found that SERCA3 is expressed most abundantly in large and small intestine, thymus, and cerebellum and in several other tissues in lower abundance. High levels of expression were also detected in various lymphoid and endothelial cells. Therefore, it was concluded that SERCA3 plays a critical role in regulating physiological processes in cells in which Ca\(^{2+}\) signaling is important. In addition, SERCA3 has been found to be present transiently together with SERCA2a in early heart development (7).

Denervation of both slow-twitch and fast-twitch muscle leads to downregulation of either SERCA2a or SERCA1, respectively. Expression of the alternative isoform was not affected by denervation. Physiological parameters, e.g., contraction time, changed consistently with reduced ATPase activity (461), although it is noteworthy that many other proteins that alter contractile properties are also changed in their expression.

Structurally, the SERCA isoforms are quite similar, although their tissue specificity indicates functional differences. Two papers deal with the functional implications of ATPase isoforms using the same technology. Different ATPase pumps were expressed in COS cells, and their activity was monitored (316, 520). Ca\(^{2+}\) dependencies were identical among SERCA1 and SERCA2a, but SERCA3 has a lower Ca\(^{2+}\) affinity and Ca\(^{2+}\) dependence. In addition, an altered pH dependence on Ca\(^{2+}\) transport was found for SERCA3. This difference is due to contributions of the nucleotide binding/hinge sequence as well as to the COOH-terminal transmembrane domains (see Fig. 11). The ATP dependence was the same in the three isoforms. SERCA2b was shown to have a lower turnover
rate for Ca\(^{2+}\) and ATP hydrolysis compared with SERCA1a and -2a.

3. Regulation

In cardiac and slow-twitch skeletal but not in fast-twitch skeletal muscle, the Ca\(^{2+}\)-ATPase is regulated by an intrinsic protein, phospholamban (472). Phospholamban inhibits the Ca\(^{2+}\) pump by reducing its Ca\(^{2+}\) affinity, which is critical for the speed of cardiac muscle relaxation. When phospholamban is phosphorylated by protein kinase A or CaM kinase after \(\beta\)-adrenergic stimulation, it dissociates from the Ca\(^{2+}\) pump and thereby the inhibition is abolished. A membrane-associated CaM kinase phosphorylates the Ca\(^{2+}\) pump (557) in addition to phospholamban in the heart muscle. Membrane attachment could occur through a newly discovered anchor protein, \(\alpha\)-KAP, which targets the CaM kinase II holoenzyme to the SR membrane (22). It was reported that the heart and slow-twitch muscle Ca\(^{2+}\) pump can be directly phosphorylated and may also be regulated by either membrane-bound CaM kinase or exogenously added CaM kinase II (183). However, other reports question the significance of this alternative activation pathway (376, 415). Similar to phos -

1. Mutations in the SERCA1 gene

A search for mutations in ATP2A1, the gene coding for SERCA1, revealed genetic heterogeneity in Brody’s
disease. Zhang et al. (569) could not find any mutation in the $\text{ATP2A1}$ gene in three patients. Odermatt et al. (377) detected three different mutations in two families that were associated with an autosomal recessive inheritance. One mutation occurred at the splice donor site of intron 3, and the two other mutations led to premature stop codons in $\text{ATP2A1}$ (377). The mutation $C592T$ causes a change of the codon CCA (Arg-198) to TGA (stop) and truncates SERCA1 at position 197 (Fig. 11). On the basis of the current model of the structure-function relations of SERCA-type $\text{Ca}^{2+}$ pumps (321), the resulting protein lacks the nucleotide and $\text{Ca}^{2+}$-binding domains and is therefore ineffective. The same result, lack of nucleotide and $\text{Ca}^{2+}$-binding domains, is predicted for the splice mutation. The third discovered nonsense mutation, $C2025A$, disrupts the $\text{Ca}^{2+}$-binding domain of the truncated protein, leaving the nucleotide binding site functional (321, 377). Thus all three mutations probably result in inactive SERCA1 proteins in vivo (Fig. 11). From these data the surprising consequence arises that the muscles of the affected individuals completely lack functional SERCA1 and that the type II muscle fibers work fairly well without the fast-twitch isoform of the SR $\text{Ca}^{2+}$ pump. The nature of possible compensatory mechanisms remains to be clarified.

2. Secondary effects on muscle fibers and muscle

Histochomical analyses of biceps brachii and quadriceps muscle biopsies showed atrophy of type II [A and B(X)] muscle fibers. A considerable fraction of type II fibers from patients with Brody’s disease was <50 $\mu$m in diameter. Such small diameters were not observed in any of the control fibers, although the control group consisted mainly of patients with peripheral neuropathies, disorders characterized by chronic partial denervation (242). In about one-half of the fibers, central nuclei were observed, but hypercontracted or necrotic fibers, ongoing regeneration or replacement of muscle tissue by connective tissue (fibrosis) was not reported (84, 242). Thus, despite of the lack of SERCA1, the fast-twitch muscle fibers of patients with Brody’s disease are protected against damage by high cytoplasmic $\text{Ca}^{2+}$. The fact that muscle stiffness, provoked by exercise, decreases after a period of rest indicates that $\text{Ca}^{2+}$ is obviously reduced to a base level by mechanisms other than $\text{Ca}^{2+}$ pumping by SERCA1.

Considering $\text{Ca}^{2+}$ in its cycle of release and uptake, a slowing down of $\text{Ca}^{2+}$ uptake into the SR can be tolerated by muscle fibers, if a normal $\text{Ca}^{2+}$ resting level is finally reached. Much worse conditions seem to be a continuous release of $\text{Ca}^{2+}$ via defective RyR or a continuous excessive $\text{Ca}^{2+}$ influx via a leaky plasma membrane.

D. Calcium Storage in the SR by Calsequestrin and Calreticulin

1. Structure and function

Calsequestrin is the major $\text{Ca}^{2+}$ storage protein in the SR of all striated muscles (for reviews, see Refs. 187, 560; see Fig. 4 for its intracellular location). It is a glycoprotein of the high mannose type located within the terminal cisternae of the SR, close to the luminal site of the functional membrane. Its role is most probably to complete the $\text{Ca}^{2+}$ cycle in facilitating the transport of $\text{Ca}^{2+}$ from the site of uptake by the $\text{Ca}^{2+}$ pump to the location of $\text{Ca}^{2+}$ release by the RyR. In addition, evidence is accumulating that calsequestrin directly modulates the release of $\text{Ca}^{2+}$ (see also sect. iii). In addition to binding $\text{Ca}^{2+}$ with low affinity (dissociation constant of 400–600 $\mu$M at 150 mM KCl and of 100 $\mu$M at 20 mM KCl) and high capacity (40–50 mol/mol), calsequestrin can be expected to neutralize the potentially negative effect of $\text{Ca}^{2+}$ on SR function (e.g., inhibition of the $\text{Ca}^{2+}$ pump) (354). Calsequestrin has an isoelectric point of pH 3.75 with more than 30% of acidic residues and binds $\text{Ca}^{2+}$ electrostatically with its acidic COOH terminus.

The two forms (fast and cardiac) of calsequestrin mainly differ in their COOH-terminal part. The cardiac form has an extended COOH terminus (residues 361–391) with 71% acidic residues and contains a second glycosylation site and several consensus phosphorylation sites for casein kinase II that have been found to be phosphorylated in vivo (59). The transcript for the fast form is expressed as the sole form in the fast-twitch muscle, as the major form in slow-twitch muscle, and is absent in the heart (12, 31, 81). In contrast, the cardiac form is the only transcript in cardiac muscle, a minor transcript in slow-twitch muscle (25% of total calsequestrin), and not expressed in fast-twitch muscles. Calsequestrin has also been found in various smooth muscle tissues and nonmuscle cells such as neurons and even in plant cells (406).

There is a two- to fourfold higher membrane RyR density in fast-twitch versus slow-twitch skeletal muscle, but physiological characteristics of the RyR channel are not significantly different and the same isoform is expressed. Because distribution of cardiac and fast-twitch muscle calsequestrin differs depending on the fiber type, it is possible that calsequestrin modulates $\text{Ca}^{2+}$ release channel physiology to some degree in a fiber-type-specific fashion (81).

2. Regulation of expression

Cardiac calsequestrin is expressed at all stages during heart development. In skeletal muscle, the fast and heart isoforms are coexpressed during neonatal development as well as in cultured myoblasts. In later...
stages, the rate of synthesis of the cardiac form decreases (388). Turning off the cardiac calsequestrin synthesis takes place between 2 and 4 wk postnataally (437). Immunocytochemical investigations of chicken embryos at the stage of early myofibrillogenesis (at day E5.5) revealed that the DHPR, the RyR, and the internal protein calsequestrin directly associated with the junctional surface of the SR are already in place at the periphery of the muscle fiber (510). During late embryonic development (E15 to E16), complex t-tubule network and internal SR transverse tubule junctions are formed, and all the components of the Ca$^{2+}$ handling system can be detected more internally. These results indicate that complexity of the Ca$^{2+}$ handling system develops in parallel to the formation of the t-tubule/SR system. The MRF myogenin seems to control the synthesis of calsequestrin in the developing muscle (12). Additionally, innervation seems to regulate calsequestrin expression. Denervation has been shown to increase its concentration as well as RyR levels but has no significant effect on nonjunctional Ca$^{2+}$-binding proteins such as the Ca$^{2+}$ pump. This suggests that the neuronal input may regulate Ca$^{2+}$ handling by altering protein levels of the junctional region of the SR (297).

3. Mode of action

Calsequestrin undergoes structural changes (increase of α-helical content, loss of hydrophobicity) induced by Ca$^{2+}$ binding (385). Secondary structure predictions suggest that the NH$_2$-terminal part contains mostly α-helix and β-sheet, whereas the COOH-terminal part contains turn and coil structures (464). Hydrophathy plot analysis indicates a low probability of membrane-spanning domains. It has been shown that calsequestrin interacts indirectly through bridging proteins with the RyR (see also sect. m4). A protein of 26 kDa named junctin, that is highly charged and basic and has binding activity for calsequestrin, was characterized (238). This protein is found in abundance in junctional membranes of cardiac and skeletal muscle SR and contains a single transmembrane domain. It shows similarity to triadin and aspartyl-β-hydrolase, which are both endoplasmic reticulum proteins with one transmembrane domain. It was suggested that junctin might be important for the formation and function of the Ca$^{2+}$ release complex. In addition, the luminal domain of triadin was found to bind calsequestrin in a Ca$^{2+}$-dependent manner and may inhibit reassociation of calsequestrin with the junctional face membrane (172). Both triadin and junctin may be calsequestrin-anchoring proteins and thereby couple calsequestrin and RyR channel activity (Fig. 4).

It is unknown so far how calsequestrin contributes to the mechanism of Ca$^{2+}$ release on a molecular level. However, several observations indicate that calsequestrin is involved in the Ca$^{2+}$ release process. It was demonstrated, for example, that calsequestrin is essential for the myotoxin α-induced Ca$^{2+}$ release from skeletal muscle SR (381). Another study shows that myotoxic drugs affect the SR protein calsequestrin and the related mitochondrial Ca$^{2+}$ storage protein calmitine by increasing proteolytic degradation of these proteins (313). In addition, in direct experiments where the open probability of the RyR was measured, it was shown that calsequestrin in the presence of millimolar Ca$^{2+}$ increases the open probability of the RyR (246).

4. Calreticulin

Calreticulin is regarded as the nonmuscle calsequestrin homolog present at highest concentration in the endoplasmic reticulum. In addition to Ca$^{2+}$ storage, this protein has been implicated in many other cellular activities including regulation of nuclear processes such as, e.g., gene transcription (reviewed in Ref. 368). During muscle differentiation, it seems that calreticulin, which is present before myotube fusion and then is replaced by calsequestrin, appears slightly before the sarcomeric myosin gene expression starts (269). In the L6 myogenic differentiation model cell line, calsequestrin was found to be expressed when differentiation is induced, whereas calreticulin levels were constant throughout differentiation except from a minor decline at a very late stage (513). This study also shows that the two proteins are coexpressed and colocalized to the SR. In addition, calreticulin but not calsequestrin was also found in the perinuclear region. Other studies where endoplasmic marker proteins were used to follow muscle differentiation confirm that the SR is basically a specialized form of the endoplasmic reticulum adapted to muscle function and that endoplasmic marker proteins coexist with the muscle-specific proteins that are responsible for efficient Ca$^{2+}$ handling (536).

5. Summary

To summarize, the low-affinity and high-capacity Ca$^{2+}$-binding protein calsequestrin is used to store Ca$^{2+}$ in the SR. By buffering Ca$^{2+}$ in the SR, the concentration of the free ionic calcium is kept low (an important role because Ca$^{2+}$ is toxic at high concentrations) and the RyR activity as well as the Ca$^{2+}$-ATPase activity are affected by the Ca$^{2+}$ concentration in the SR. Calsequestrin is produced in a fast and a cardiac isoform and is considered the muscle cell homolog of calreticulin, which seems to control other cellular activities in addition to its Ca$^{2+}$ storage function in the ER and SR.
E. Potential Role of Other Calcium-Binding Proteins Not Directly Involved in the Calcium Cycle: Calpain, Sorcin, Annexins, S100 Proteins, Myosin Light Chains, α-Actinin, and Calcineurin

1. Calpains

A) Structure and Mode of Activation. Calpains are cysteine proteases that are Ca\(^{2+}\) dependent (for review articles, see Refs. 282, 507, 508). Because it seems that these proteases have a profound effect on crucial components of the Ca\(^{2+}\) cycle, including the RyR, and the troponin complex, they are discussed in some detail. Calpain consists of a large and a small subunit of 80 and 30 kDa, both of which bind Ca\(^{2+}\) in CaM-like structures with 4 EF-hand domains (Fig. 12). Experimental data suggest that activation of calpains by Ca\(^{2+}\) involves dissociation of the two subunits (564). At present, however, it is unclear how the activity of calpain is regulated in the cell. It seems that tight control is absolutely necessary to prevent unwanted damage by proteolysis. One important aspect in regulation is that activated calpains are very unstable. Deviations from normal calpain levels may be lethal as indicated by the fact that manipulation by overexpression or inhibition is at present difficult if not impossible. It is still mysterious how the moderate Ca\(^{2+}\) dependence of calpain found in vitro is modified in the cell to adapt to physiological conditions. Suzuki et al. (508) propose that the first step in activation is a Ca\(^{2+}\)-dependent translocation to the cell membrane in the heterodimeric form, since the small subunit is responsible for membrane interaction by its hydrophobic NH\(_2\) terminus (Fig. 12). Calpain can then be activated by phospholipids. This leads to autolysis (with a large subunit of 76 kDa and a small subunit of 18 kDa being the active forms) at the cell membrane, since phospholipids decrease the Ca\(^{2+}\) requirement of the enzyme. Alternatively, activation can also be achieved by a Ca\(^{2+}\)-dependent dissociation of the small from the large subunit. Possibly cellular factors not yet identified are involved in calpain activation. The dissociated 80-kDa subunit is enzymatically active (508). For further details of the individual steps, see Reference 508. Note that Ca\(^{2+}\) is required for all steps. PIP\(_2\), phosphatidylinositol 4,5-bisphosphate. (Figure kindly provided by Dr. Suzuki, Tokyo, Japan.)
B) Regulation by Inhibition. The activity of calpains is believed to be regulated by the specific inhibitor calpastatin, a protein with a molecular mass of 110 kDa. Ultrastructural localization of calpastatin as well as biochemical analysis have revealed that a good correlation in calpain and calpastatin intracellular distribution exists (375). Enzyme and inhibitor are found in the sarcolemma with the adjacent cytoplasm, the myofibrils, the mitochondria, and the nuclei but not in lysosomes and the intermyofibrillar cytoplasm. No clear muscle fiber type specificity was found. m-Calpain was found at higher concentration in slow-twitch muscle (masseter, composed exclusively of oxidative fibers) and μ-calpain at higher concentration in a muscle containing a mixture of fiber types (with 65% glycolytic fibers), whereas calpastatin was found at similar levels in the two types of investigated porcine muscles (254).

The μ- and m-calpains are localized within the Z disks of skeletal muscle fibers (85). Immunofluorescence showed that both calpains and calpastatin are approximately two times more abundant at the Z disk of myofibrils than in the I-band region. Denervation, fasting, and refeeding increase the concentration of the enzyme and the inhibitor but do not change their distribution (277). Calpastatin affects conversion from inactive to active calpain at the membrane, translocation of active and inactive calpain, and the activity of calpain itself. However, the exact physiological role of calpain inhibition by calpastatin is not understood so far.

Calpain does not have a defined amino acid substrate requirement. It seems that protein degradation is carried out to a limited degree and on a regulatory level (only at a limited range of targets) in contrast to digestive proteases with broad target specificities. It has been shown that calpain cleaves protein substrates in between rather than within functional domains leading to alteration of protein functions. Therefore, it has been concluded that calpains play an important regulatory role in cellular functions.

Some years ago, two novel tissue-specific calpain species (n-calpains) have been discovered. One is skeletal muscle specific and the other was found in the smooth muscle (stomach) (484). The skeletal muscle specific isoform (p94) has a unique feature in that its half-life is extremely short (25 min). Its message is found at ~10-fold higher levels compared with the μ- and m-forms. By elegant mutagenesis work (reviewed in Ref. 484), it was shown that a short sequence adjacent (NH2 terminal) to the CaM-like domain of p94 (named IS2) is responsible for the short half-life. This form is absent in all other calpain forms. P94 exists in the cytosol, as the μ- and m-forms, but is more concentrated in the nucleus. It was suggested that the function of p94 is to regulate MRF such as MyoD and myogenin and thereby regulating growth and differentiation of muscle cells. The nuclear localization signal lies in the region (IS2), which is also responsible for fast degradation. Interestingly, although the calpain small subunit is known to be important for the regulation of calpain, it was not found to be associated with p94 as analyzed by the two hybrid systems. So far, all tissue-specific forms (unlike the ubiquitous forms) do not seem to dimerize with the small subunit (reviewed in Suzuki et al., Ref. 508). However, connectin (titin), a giant muscle protein which connects M and Z lines of muscle sarcomere, was found to interact with p94 in the IS2 region, indicating that p94 might be regulated by connectin (484).

Genetic and physical studies of LGMD2A, an autosomal recessive form of limb girdle muscular dystrophy, lead to the discovery that n-calpain p94 is the affected gene in this disease (419) (see also sect. v). The mutations destroy the proteolytic activity of p94, and the lack of protease activity rather than an increase is the primary cause of the disease. Calpain might also play a significant role in myonephropathic metabolic syndrome (MNMS), a serious muscle reperfusion injury associated with acute renal failure, since administration of a calpain antagonist prevented MNMS (523). Dystrophin-deficient mouse muscles (mdx) contain higher calpain concentrations compared with controls (487). μ-Calpain had an increased activity in mdx muscle due to autoproteolysis, which returns to control levels during regeneration. Muscle exercise results in an increase of nonlysosomal Ca2+ protease activity, possibly promoting exercise-induced muscle damage or fatigue (23).

C) Regulation of Expression and Involvement in PKC Signaling. Sequence similarities in the 5′-flanking regions of the genes for the m-calpain large subunit and the small subunit indicate a similar regulation. It has been shown on the one hand that phorbol esters stimulate the expression of m-calpain, suggesting that calpain could be regulated by PKC. In addition, 12-O-tetradecanoylphorbol-13-acetate (TPA) causes translocation of PKC as well as m-calpain. Downregulation of the PKC in the membrane fraction on the other hand has been shown to be blocked by calpastatin (205). It has been shown that m-calpain and p94 rather than μ-calpain, which seems to have rather housekeeping functions, downregulate the level of PKC and transcription factors (200, 485).

The second messenger NO has been proposed to play a role in relaxation of fast-twitch muscle fibers (266). NO inactivates m-calpain at neutral pH (348). In contrast, μ-calpain activity was affected by NO only if the pH was shifted to acidic values, a condition which does not allow inhibition of m-calpain by NO. Therefore, it might be speculated that NO may selectively affect calpain isoforms depending on the hydrogen ion concentration in contracting muscles under physiological and pathological conditions. Interestingly, the NO synthase present in highest abundance in the muscle is the Ca2+/CaM-dependent type (42). Therefore, calpain activity might be regulated
by Ca$^{2+}$ through CaM/NO synthase (for CaM targets, see sect. II A and Table 2).

D) Effects on muscle proteins. Several studies have shown that calpains initiate turnover of myofibrillar proteins by specifically cleaving them and releasing large polypeptide fragments from the myofibrils (reviewed in Ref. 161). This process seems to be required for muscle growth. In addition, calpain-mediated cleavage of cytoskeletal proteins such as filamin might be important for myogenic differentiation (279). m-Calpain concentration and activity increase during early myogenic differentiation, whereas the level of calpastatin remains similar during this period and μ-calpain was not detected, indicating that m-calpain is responsible for Ca$^{2+}$-dependent proteolytic activity during muscle cell differentiation in culture (75). A regulatory activity of calpain on DHPR function has also been proposed. μ-Calpain specifically cleaves a 37-kDa fragment containing the major CaM-dependent phosphorylation site from the COOH terminus of the α1-subunit of the L-type Ca$^{2+}$ channel and therefore may regulate the cAMP dependency of DHPR (86).

m-Calpain cleaves the RyR either when purified or in the triad membrane, resulting in peptides of 160 and 410 kDa which are then further cleaved by the same enzyme finally leading to polypeptides of 70, 140, and 200 kDa. RyR cleavage is inhibited by CaM. Because the RyR contains PEDST (proline, glutamic acid, aspartic acid, serine, threonine-rich) protease cleavage sequences, it was suggested that RyR is a PEDST-type calpain substrate. These substrates are usually cleaved near CaM binding sites, and therefore, CaM protection of cleavage can be expected (40). Some years ago, on the basis of measurements of Ca$^{2+}$ dependence and specific inhibitors, it was suggested that μ-calpain associates with the functional SR and cleaves the RyR into two fragments (375 and 150 kDa) at a PEDST site (471). Similarly, as found in other studies, this cleavage stimulates Ca$^{2+}$ efflux but had no effect on other features of the RyR.

Calpains also seem to specifically affect the troponin protein complex (98). μ-Calpain was at least 10 times more effective than m-calpain in degrading TnI and TnT in vitro and in situ, whereas TnC was resistant to both forms. PKA phosphorylation reduced the sensitivity of TnI toward μ-calpain degradation. On the other hand, PKC phosphorylation of TnI increased the proteolytic degradation of TnI.

2. Sorcin

Sorcin has been identified as a calpain-like protein of 22 kDa in cells selected for drug resistance (reviewed in Refs. 345, 530). It has been reported to be abundant in skeletal, heart, and smooth muscle but occurs also in nonmuscle cells. In the presence of Ca$^{2+}$ during protein isolation, sorcin was found in the insoluble fraction, whereas in the absence of Ca$^{2+}$, sorcin was soluble, indicating Ca$^{2+}$-dependent translocation (346). This protein has 2 EF-hands (at least one with high Ca$^{2+}$ affinity) and two atypical Ca$^{2+}$-binding domains as well as phosphorylation sites for PKC and CaM kinase (530). Sorcin has been found to be associated with the RyR, suggesting a role in intracellular Ca$^{2+}$ release (347). A function of sorcin in Ca$^{2+}$ flux through the SR is also suggested by the observation that transfection of sorcin into fibroblasts elicits a musclelike, caffeine-stimulated release of Ca$^{2+}$ from intracellular stores (345).

3. Annexins

Annexins are a diverse family of Ca$^{2+}$-dependent phospholipid binding proteins with a wide distribution and are most likely involved in many different cellular activities, e.g., cellular vesicle traffic and exocytosis (for reviews, see Refs. 54, 55, 76). The common feature of annexins is a conserved domain that is repeated four or eight times and that is responsible for phospholipid-dependent Ca$^{2+}$ binding that varies over the low and high micromolar range. A potential role in muscle physiology has been ascribed to annexins VI and VII (113, 114, 491), which both have voltage-dependent Ca$^{2+}$-channel activity (92). Annexin VI has been shown to be associated with intracellular organelles, especially those involved in sequestration or release of Ca$^{2+}$ (105, 184). This protein was shown to modify, in a Ca$^{2+}$-dependent manner, the behavior of the SR Ca$^{2+}$ release channel in an artificial bilayer by increasing both open probability and mean open time (97). By an overlay technique it was demonstrated that annexin VI interacts with PKC-β in the skeletal muscle, and it was concluded that annexin VI plays a role in regulating the activity of the Ca$^{2+}$-dependent PKC-β (457). Annexin VII also known as synexin has been found at the plasma membrane and the t-tubule system in skeletal muscle (467), and it was suggested that synexin may be involved in EC coupling in the skeletal muscle. Patients with muscular dystrophy and the mdx mouse showed redistribution of annexin VII into the cytoplasm most likely due to disintegration of the membrane-linked cytoskeleton. In addition, because of membrane lesions, this protein can also be found in the extracellular space (466).

4. S100 proteins

S100 proteins are small Ca$^{2+}$-binding proteins containing two EF-hand Ca$^{2+}$ domains. So far more than a dozen different genes for this protein family have been found, all exhibiting unique spatial and temporal expression patterns. These proteins have been implied to play a role in many different biological activities such
as cell cycle progression, cell differentiation, tumorgenesis, cytoskeletal reorganization, metabolism, neurotransmission, and many other functions (reviewed in Refs. 100, 448, 449, 572). S100A1 is especially interesting with respect to muscle physiology, since it has been shown to be present at high concentration in slow-twitch muscle fibers (574). It colocalizes with the SR (575) in skeletal muscle, whereas in the heart, localization was found in the sarcolemma, the SR, the myofilaments, and the nuclei (101, 177). S100A1 was localized in the mouse soleus muscle at polysomes, the SR, the plasma membrane and in pellicle around lipid droplets, the outer membrane of mitochondria, and thin and thick filaments by electron microscopy (176, 571). Recently, it was shown that S100A1 activates $^{3}$Hryanodine binding activity to the RyR at nanomolar Ca$_{2+}$ concentrations and at physiological S100 protein levels (521). In addition, it was found that S100A1 activates the open probability of this Ca$_{2+}$ channel severalfold, and direct interaction between S100 and the RyR was demonstrated by the optical sensor BIACore. Interestingly, S100A1 was found to bind and specifically activate the protein kinase twitchin in a Ca$_{2+}$-dependent manner in vitro (186). Twitchin is a giant myosin-associated protein. Because protein kinase activity is enhanced by S100A1 up to 1,000-fold, it was suggested that this Ca$_{2+}$-binding protein may be the physiological activator of twitchin. In addition, glycogen phosphorylase exhibits S100A1 specific regulation (reviewed in Ref. 573).

5. Myosin light chains

In sarcomeric myosin (myosin II), four light-chain molecules are bound noncovalently to two heavy chains. Two subfamilies of myosin light chains with molecular mass of 16–22 kDa exist: essential light chains (ELC) (reviewed in Ref. 450) and regulatory light chains (RLC) (reviewed in Ref. 451). There exists considerable tissue and muscle fiber type specificity of isoform expression. ELC and RLC contain four putative EF-hand domains and belong to the CaM superfamily. All four EF hands of the ELC have lost their Ca$_{2+}$-binding activity during evolution and therefore are not relevant for further discussion in the context of Ca$_{2+}$ regulation. RLC have maintained one active Ca$_{2+}$ domain. In vertebrates, CaM-dependent reversible phosphorylation of RLC regulates smooth muscle and nonmuscle cell contraction (2, 73), whereas RLC have no role in directly triggering striated muscle contraction but rather act in modulation of contractile activity. The Ca$_{2+}$/Mg$^{2+}$ site of RLC has an apparent affinity for Ca$_{2+}$ in the range of dissociation constant $10^{-5}$ M in the presence of millimolar Mg$^{2+}$ concentration (543). Therefore, this site is not involved in muscle contraction and thought to be Mg$^{2+}$ filled during contraction. It may be occupied by Ca$_{2+}$ during prolonged Ca$_{2+}$ elevation, for example, during tetanus.

6. α-Actinin

α-Actinin is an EF-hand protein belonging to the F-actin binding proteins such as spectrin and dystrophin. It is associated at the actin filament system of the muscle but is also present in nonmuscle cells as a component of the cytoskeleton (373). α-Actinin is present in the Z disks and thought to connect the ends of the parallel and antiparallel arrays of actin filaments (344). Binding of Ca$^{2+}$ to EF-hands regulates the activity of nonmuscle α-actinin, whereas the EF-hands in the muscle α-actinin are incomplete and α-actinin cross-linking is Ca$_{2+}$ independent (reviewed in Ref. 373).

7. Calcineurin

Calcineurin is a Ca$^{2+}$/CaM-dependent phosphatase consisting of a catalytic subunit and a regulatory subunit that is a CaM-like protein (171, 255). Because of its narrow substrate specificity, calcineurin is a candidate for an important signal transmission molecule. Calcineurin binds to and is inactivated by the immunosuppressant drugs cyclosporin and FK506 (308), which themselves bind to cyclophilin and FK506 binding protein, respectively. It was shown that RyR and IP$_{3}$ receptor bind to the FK506 binding protein and that the immunosuppressant drug FK506 disrupts this association (Fig. 4). On the other hand, FK506 stimulates the binding of FK506 binding protein to calcineurin. FK506 binding to the Ca$^{2+}$ release channel proteins alters their Ca$_{2+}$ transport properties (61, 518). In the absence of the FK506 binding proteins, the Ca$_{2+}$ channels become "leaky" and therefore net accumulation of Ca$_{2+}$ into RyR- or IP$_{3}$-R-gated stores is diminished. By anchoring calcineurin to the IP$_{3}$ Ca$_{2+}$ channel through the FK506 binding protein, the phosphorylation state of the IP$_{3}$ channel is altered and thereby Ca$_{2+}$ flux activity is modulated (60) (for a discussion on RyR regulation, see also sect. II).

Calcineurin is known to be directly involved in cytokine gene expression in B and T cells in a Ca$^{2+}$/CaM-dependent manner (413). Dephosphorylation of nuclear factor activated T cells (NFAT) by calcineurin enables the transcription factor to translocate from the cytoplasm to the nucleus where it activates a variety of genes.

Recently, it has been proposed that calcineurin could play a similar role in the muscle as a mediator of the Ca$^{2+}$-dependent signal transmission process determining fiber type gene expression (66). It is known that tonic motor nerve activity is needed for slow muscle specific gene expression, whereas brief bursts of neural activity with long periods of quiescence determines the fast muscle specific phenotype. Slow muscle fibers are characterized by a relatively high Ca$_{2+}$ concentration, whereas
Ca\textsuperscript{2+} levels in fast muscles are low. In T cells, it could be shown that activation of the calcineurin-dependent signaling process needs sustained Ca\textsuperscript{2+} elevation as found in the slow muscle, and short transient Ca\textsuperscript{2+} elevations as found in fast muscles are not sufficient to activate this system (99, 519).

Skeletal muscle hypertrophy and a switch to glycolytic metabolism of differentiated myotubes induced by growth factors such as insulin-like growth factor or insulin is mediated by calcineurin in a Ca\textsuperscript{2+}/CaM-dependent fashion (468). During muscle hypertrophy, calcineurin induces the expression of the transcription factor GATA-2, which associates with calcineurin and a specific isoform of the transcription factor NFATc1 (366). Cyclosporin prevents muscle hypertrophy and muscle fiber type conversion associated with functional overload in vivo, indicating that postsurgical muscle wasting and weakness is due to the administration of the immunosuppressant cyclosporin (111). Calcineurin activity has been shown to selectively upregulate slow fiber specific gene promoters, and inhibition of calcineurin leads to a slow-to-fast fiber transformation (66). The transcripational activation of slow type specific genes is mediated by the NFAT and MEF2 transcription factors. On the basis of these findings, it was speculated that calcineurin could become clinically relevant since modification of its activity could be used to transform fast into slow muscle, the latter being less affected in dystrophy. This is a good example of how the Ca\textsuperscript{2+} signaling system may directly specify fiber type-specific gene expression and thereby determine the phenotype of a muscle fiber.

8. Summary

In summary, many proteins found in muscle cells have the ability to bind Ca\textsuperscript{2+} with high or moderate affinity and therefore affect and/or modify the primary Ca\textsuperscript{2+} cycle, finally leading to altered muscle performance. Most of the proteins discussed (sorcin, annexins, S100 proteins) have no clear functional role in muscle physiology, and therefore, in most cases a list of known features is presented. However, much is known about the ubiquitous Ca\textsuperscript{2+} receptor CaM (discussed in sect. III in the context of RyR regulation) and about the Ca\textsuperscript{2+}-dependent protease calpain. The latter protein selectively degrades several key proteins in the calcium cycle and is involved in several muscle diseases. Most remarkable, mutations in a gene for a newly discovered calpain isoform (p94) are responsible for one form of limb girdle muscular dystrophy (LGMD2A).

![Diagram of dystrophinopathies and related diseases](https://physrev.physiology.org/content/1247/1/1247/F13)

**Fig. 13.** Presumed pathomechanism of dystrophinopathies and related diseases. Schematic drawing of a part of a muscle fiber (longitudinal section) with sarcolemma, sarcoplasmic reticulum, nucleus, and mitochondrion. In normal muscle, dystrophin connects the cytoskeleton (F-actin) via a complex of glycoproteins, dystroglycans (DG), sarcoglycans (SG), and sarcospan (white rectangle) to laminin in the extracellular matrix. Additional proteins, syntrophins and dystrobrevin, are bound to the COOH terminus of dystrophin. Deficiency of dystrophin in Duchenne muscular dystrophy (DMD, 1) and the X-linked muscular dystrophy of the mouse (mdx, 1) leads to a loss of connection of the muscle fibers and the extracellular matrix. The same defect, loss of connection between cytoskeleton and extracellular matrix, occurs by deficiency of sarcoglycans (α, β, γ, δ) that cause several forms of limb girdle muscular dystrophy (LGMD, 2). Mutations in the laminin α2-gene can cause congenital muscular dystrophy (CMD, 3). At least in dystrophinopathies the sarcolemma is characterized by leakiness, allowing the exchange of macromolecules between the extracellular fluid and the cytoplasm. The lesions also allow an excess of Ca\textsuperscript{2+} influx. The overactivity of plasma membrane Ca\textsuperscript{2+} channels may aggravate Ca\textsuperscript{2+} influx. A compensatory Ca\textsuperscript{2+} uptake into the SR and the mitochondria follows until, finally, toxic Ca\textsuperscript{2+} overload causes a variety of pathological changes including necrosis and apoptosis (see Fig. 14).
V. ALTERATION IN CALCIUM HANDLING IN DYSTROPHINOPATHIES AND RELATED DISEASES

Muscular dystrophies are a group of hereditary diseases characterized by muscle fiber necrosis and progressive muscle wasting and weakness. The genetic defects underlying these muscle diseases are not directly related to proteins of the calcium cycle (Table 2), but excessive calcium influx into muscle fibers or disturbed intracellular calcium signaling are presumably involved in the pathomechanisms of muscle dystrophies. As shown in earlier sections of this review, Ca\(^{2+}\) levels in the cytosol and the SR play important roles in the regulation of Ca\(^{2+}\) cycle molecules. Therefore, Ca\(^{2+}\) handling in muscle dystrophy is discussed in some detail. The question of whether pathological alterations of Ca\(^{2+}\) handling are an early or a late step in the disease mechanism has to be clarified for the different muscle dystrophies. In addition to the fact of a better understanding of skeletal muscle calcium control, these results can also have important consequences for therapeutic approaches.

Many of the dystrophies, but not all, are diseases of the dystrophin-glycoprotein complex and are today classified as dystrophinopathies. The human Duchenne muscular dystrophy (DMD) is the best known since it is one of the most frequent genetic human diseases (~1:3,500 male births; Ref. 202). The primary defect in DMD is the lack of dystrophin, a 427-kDa subsarcolemmal cytoskeletal protein. In normal muscle it links the cytoskeleton (actin) via a complex of membrane proteins (dystrophin-associated glycoproteins, e.g., dystroglycans and sarcoglycans) to laminin in the extracellular matrix (Fig. 13) (62). The lack of dystrophin is due to mutations in the dystrophin gene which is extraordinarily large (>2,300 kB) and localized on the X chromosome at Xp21 (Table 2). More than 1,500 deletion breakpoints have been detected in the human dystrophin gene only in European populations (83). Several animal models of muscular dystrophy exist including the X-linked dystrophy of the mouse, mdx (49, 202). Today it seems clear that all DMD patients lack dystrophin independent of the underlying mutation, and it is believed that the lack of dystrophin causes the phenotype of dystrophy.

1. Clinical course of DMD

In DMD, the first clinical symptoms are usually observed before the age of three in affected boys who show a delay in reaching developmental milestones such as running and climbing. Before age 6 their gait becomes unsafe and waddling, and in the same period of time hypertrophy and pseudohypertrophy of calf, gluteal, quadriceps, and other muscles develops. Between 6 and 11 years of age muscle strength declines steadily, leaving boys wheelchair bound from about age 11. Death occurs before age 30 by respiratory failure (as a consequence of weakness of respiratory muscles) often in combination with respiratory infection or by cardiac failure (117). The histological analysis of DMD muscle characteristically reveals eosinophilic hypercontracted muscle fibers, necrotic fibers, ongoing muscle regeneration, and the proliferation of fibroblasts within muscle tissue. The replacement of muscle tissue by connective tissue (fibrosis) is the major cause of muscle weakness. Degeneration and regeneration of muscle fibers does also occur in mdx muscle, but fibrosis is seen to a much smaller extent and has a late onset (202).

2. Early steps in the pathomechanism

Although the primary defect in DMD has been known for more than 10 years (201), neither the function of dystrophin nor the pathogenic mechanism of muscle dystrophy is sufficiently clarified. Two different, not necessarily exclusive, hypotheses have been put forward to explain the pathological changes caused by dystrophin deficiency, i.e., the “calcium hypothesis” and the “leaky membrane hypothesis” (155, 202, 330, 500).

3. The calcium hypothesis

This hypothesis is based on early reports of Ca\(^{2+}\) accumulation in DMD muscle fibers. An alizarin red stain, a histochemical stain for Ca\(^{2+}\) deposits, showed increased numbers of positive fibers in DMD muscle compared with controls. Necrotic as well as nonnecrotic fibers were alizarin positive (34). The total Ca\(^{2+}\) content of DMD muscle biopsies was found to be elevated compared with controls by a factor of 2.4 (228). A similar Ca\(^{2+}\) increase was found in adult mdx muscle at all tested stages (157). The mechanism of Ca\(^{2+}\) entry and its connection to dystrophin deficiency are still controversially discussed. An increased activity of plasma membrane Ca\(^{2+}\) channels in myotubes of DMD and mdx origin has been reported (138, 207). Elevated resting levels of intracellular free Ca\(^{2+}\), detected with fluorescent indicators, were observed in DMD myotubes (355) and mdx muscle fibers (525). These data suggested a direct or indirect involvement of dystrophin in the muscular Ca\(^{2+}\) homeostasis and a close correlation between the lack of dystrophin and increased Ca\(^{2+}\) influx. In later studies, these findings could not be confirmed. Several groups showed unchanged free Ca\(^{2+}\) resting levels and Ca\(^{2+}\) transients in cultured myotubes of DMD and mdx origin (407, 428) and in mdx muscle fibers (146, 185, 407). Interestingly, hyposmotic stress, a means to mimic mechanical stress, induced Ca\(^{2+}\) transients in myotubes (407), and they were more pronounced in mdx myotubes (298). Contracting but not resting DMD myotubes, cocultured with...
4. The leaky membrane hypothesis

According to the leaky membrane hypothesis, the sarcolemma of dystrophin-deficient muscle fibers is more susceptible to lesions. This hypothesis was developed on the observation that increased concentrations of muscle-specific cytoplasmic proteins are present in the serum of DMD patients (117, 133, 568) and mdx mice (157, 331) even before the onset of muscle fiber degeneration. Creatine kinase (CK) activity can be 100-fold higher in serum of DMD patients compared with controls (568). In addition to CK, many other muscle-specific cytoplasmic proteins as pyruvate kinase, myoglobin (133), and PV (235) are found in the serum when skeletal muscle is dystrophin deficient. The release of cytoplasmic enzymes from dystrophin-deficient muscle (Fig. 13) has been reproduced with isolated muscle fibers (341) and cultured mdx and DMD muscle (342). Not only efflux, but also influx of molecules into dystrophin-deficient muscle fibers has been shown in vitro and in vivo. Preparations of isolated mdx diaphragm exposed to eccentric contractions showed much higher intake of procion orange than control muscle (394). This result was later confirmed with extensor digitorum longus muscle from 40-day-old but not from 2-wk-old mdx mice (331). Matsuda et al. (329) demonstrated that uptake of molecules into mdx muscle fibers occurs in vivo. Intravenous injection of Evan’s blue resulted in staining of mdx muscle fibers, indicating the uptake of the dye, which is in plasma bound to albumin, into dystrophin-deficient muscle fibers (329, 499, 500). Evans blue does not cross into skeletal muscle fibers in normal mice (500). Taken together, a bidirectional flow of molecules between the cytoplasmic and the extracellular space seems to be characteristic for dystrophin-deficient muscle in vitro and in vivo. This exchange of molecules occurs during normal mechanical muscle activity and is aggravated by mechanical stress.

5. Insights from the diseases related to dystrophin-associated proteins

Recent findings about the roles of laminin, a component of the extracellular matrix of skeletal muscle, and the dystrophin-associated glycoproteins (DAG), α- and β-dystroglycan and α-, β, γ, and δ-sarcoglycan (499) gave new insights in dystrophin function. Mutations in the mentioned proteins can also cause muscle dystrophy (Table 2). An autosomal recessive form of muscular dystrophy (severe childhood autosomal recessive muscular dystrophy, SCARMD) is associated with mutations in the α-sarcoglycan (former name: adhalin) gene (429, 499). The genes coding for β, γ and δ-sarcoglycan (Fig. 13) cause several forms of limb-girdle muscular dystrophy (LGMD) when affected by mutation (LGMD2C, γ-sarcoglycan (374); LGMD2E, β-sarcoglycan (35); LGMD2F, δ-sarcoglycan (372)). Congenital muscular dystrophy (CMD) is a clinically heterogeneous group of muscular dystrophies often with early onset. In one form mutations in the LAMA2 gene, coding for the laminin α2 chain (Fig. 13), the natural extracellular ligand of α-dystroglycan, are responsible for this form of muscular dystrophy in humans (Table 2; Ref. 192). The homologous murine models are the dystrophia muscularis (dy/dy) and the milder allelic variant, the dy2J/dy2J mouse (505). Compared with controls, muscle fibers of dy animals showed on average a two- to fourfold increase of their free cytoplasmic Ca^{2+} concentration as determined with the fluorescent indicator.
fura 2 (549). Obviously, also laminin defects can cause impairment of muscular Ca\(^{2+}\) homeostasis. However, the pathomechanisms leading to intracellular Ca\(^{2+}\) increase and to muscle dystrophy of laminin-deficient animals seem to be different from those of mdx mice, because only the latter showed staining of muscle fibers after intravenous injection of dyes (500). From these observations it follows that the sarcolemma of dy and dy2J muscle fibers is fairly impermeable for macromolecules and that mechanisms other than membrane leakage are responsible for the Ca\(^{2+}\) increase in dy muscle fibers. This is a remarkable difference between dy and mdx fibers, because dystrophin and laminin \(\alpha 2\) chain are both associated with the same protein complex.

Recently, the gene defect underlying LGMD2B has been discovered (21). The gene shows no homology to any known mammalian gene. Thus localization and function of the protein product, called dysferlin, remain unclear today.

It can be concluded that the function of the dystrophin-glycoprotein complex is to anchor the muscle fibers in the extracellular matrix. A loss of function of any of the proteins involved in this anchor system (dystrophin, DAG, and laminin) can cause muscle dystrophy. In addition, dystrophin itself, by forming a subsarcolemmal membrane-associated network of filaments, mechanically stabilizes the muscle fiber membrane (341, 342, 389).

6. The mechanical hypothesis: an integrated view

If we return to the pathophysiology of dystrophin-deficient muscle, the above-mentioned calcium hypothesis and the leaky membrane hypothesis may be integrated to a “mechanical hypothesis.” A higher fragility of the plasma membrane during mechanical activity seems to be the direct physiological consequence of the lack of dystrophin. This increased fragility results in short-lived membrane lesions of limited size, which allow the efflux of cytoplasmic molecules from the cell and also the influx of molecules into the sarcoplasm. The efflux of cytoplasmic components is probably an indicator of muscle membrane damage rather than great pathophysiological importance. Among the molecules that enter the muscle fibers, Ca\(^{2+}\) are thought to be those with the greatest pathogenic consequences (155). Increased influx of Ca\(^{2+}\) into the subsarcolemmal space can lead to the activation of degradative enzymes and overload and dysfunction of Ca\(^{2+}\) cycle and storage systems. This results in damage of the sarcolemma from interior, impairment of mitochondrial function, and modulation of intracellular signaling pathways (Figs. 13 and 14).

7. Calcium as a pathogenic factor in DMD

Because membrane lesions are of limited size in the beginning of the necrotic process and Ca\(^{2+}\) is quickly bound by target proteins, the early consequences of Ca\(^{2+}\) influx will probably be restricted to the subsarcolemmal space of the muscle fibers. A key factor may be an increased calpain activity (see sect. ivE, calpains). Possible substrates of calpains are the membrane cytoskeleton, the Ca\(^{2+}\)-ATPase of the plasma membrane, and ion channel proteins. The Ca\(^{2+}\) pump located in the plasma membrane is a preferred substrate of calpain in erythrocytes (439), and if attacked in dystrophin-deficient muscle, this calpain action would, in addition to provoking an excess of Ca\(^{2+}\) influx, disturb an important extrusion pathway. Another pathway of Ca\(^{2+}\) influx, in addition to that mediated by membrane lesions, was pointed out by Turner et al. (524). They demonstrated that proteolytic cleavage of plasma membrane Ca\(^{2+}\) channels can lead to increased openings of these channels (524). This could mean a loop of positive feedback of Ca\(^{2+}\) influx, Ca\(^{2+}\)-dependent proteinolysis, and increased Ca\(^{2+}\) influx (Fig. 14). Thus dystrophinopathies are primarily diseases of the sarcolemma.

A weakened sarcolemma can in the long range not prevent Ca\(^{2+}\) from entering the interior of mdx muscle fibers. This can have severe consequences for muscle function and structural integrity. Experimentally induced short-term elevation of intracellular Ca\(^{2+}\) into the micromolar concentration range was shown to inhibit or even abolish excitation-contraction coupling in toad and rat muscle fibers (280). This effect does not require structural damage and could contribute to muscle weakness in various dystrophies before fiber damage occurs. For DMD muscle fibers, weakness has been quantified on the cellular physiological level. The maximum tension development ability was found in <20% of type IIA DMD fibers compared with control (128). Further consequences of dystrophin deficiency are the following: increased protein degradation, mitochondrial damage (313), myofibrillar damage, dysfunction of the Ca\(^{2+}\)-ATPase of the SR (240), necrosis, and the activation of apoptotic pathways (444, 517). These processes are introducing the final steps of cell damage and cell death of DMD and mdx muscle fibers (Fig. 14).

Increased protein degradation was found in mdx muscle (525), and it was argued that increased degradation results from the elevated Ca\(^{2+}\) levels found in dystrophic muscle. MacLennan and Edwards (322) showed that increased protein degradation is compensated by an increased protein synthesis. Furthermore, they found elevated protein turnover in mdx muscle without a net loss of functional protein. This finding is consistent with the observation that mdx mice, as young DMD patients, show muscle hypertrophy (181, 202) and increased muscle strength at most stages. It is also in agreement with the finding that mdx mice are more sensitive to fasting than wild-type mice (194), i.e., a lack of amino acids is more severe in combination with a higher protein turnover. The following potential mediators of hypertrophy of dystro-
phosphodistrophin-deficient muscle have been discussed: an elevated basal level of adenylate cyclase activity and elevated cytoplasmic Ca\(^{2+}\) levels. Ca\(^{2+}\) signaling via CaM (15) or calcineurin (468) is a good candidate for a signal-transduction pathway into the nucleus. Furthermore, an increase of c-myc expression has been suggested. In this context also, an increased synthesis and secretion of insulin like growth factors (IGF-I and IGF-II) and following autocrine and paracrine stimulation of muscle fibers has been discussed (181). Recently, it was indeed shown that IGF-I induces skeletal myocyte hypertrophy through calcineurin in association with transcription factors GATA-2 and NFATc1 (366).

The increase in protein degradation in mdx muscle is probably due to the higher activity of calpains, Ca\(^{2+}\)-binding neutral proteases (78) (see also sect. ivE, calpains), in dystrophic muscle (487), but it is not clear how a general increase in protein turnover can initiate the dystrophic process. Using immunohistochemical staining, Kumamoto et al. (278) showed an increase in calpain especially in the myofibrillar area (Z disks) in atrophic DMD muscle fibers but not in morphologically intact fibers. This indicates that an increased calpain content of dystrophin-deficient muscle fibers is probably not an early event in the dystrophic process.

An alternative pathway of cell damage involving mitochondrial dysfunction has been suggested for dystrophin-deficient muscle (313, 553). Mitochondrial mRNA have been found to be downregulated in mdx muscle (148), and a lack of calmitine, a Ca\(^{2+}\)-binding protein present in mitochondria, was shown in DMD muscle (see sect. ivD). However, it is not clear whether mitochondrial dysfunction and energy depletion are critical and early steps in the dystrophic process.

8. Different sensitivity of the muscle fiber types

Some interesting data with regard to the pathomechanism of dystrophy have been obtained from comparisons of the fiber type specificity of muscle damage. In the human DMD, the fast-twitch glycolytic type IIB (IX) muscle fibers were found to be preferentially affected (128, 546). The authors suggested that the burstlike stimulation pattern and the high force production of type IIB (IX) fibers are less compatible with dystrophin deficiency. A similar result was found in mdx muscle. Here, the authors described that small-caliber fibers better tolerate dystrophin deficiency than the bigger IIB (IX) fibers (241). The observation that extraocular muscles are not affected in DMD (252) is in agreement with the discussed observations. The fibers of extraocular muscles are characterized by small diameters and very fast reuptake into the SR after activation.

The effect of reduced electrical activation of mdx muscle was tested in vivo by breeding a double-mouse mutant, the gad-mdx mouse. This mutant showed that a peripheral neuropathy leading to muscle fiber atrophy (gad mutation) has a positive influence on muscle fiber integrity of dystrophin-deficient (mdx) muscle (504). From these data it was concluded that a generally reduced stimulation of muscle and the lack of high-frequency discharges are beneficial for dystrophin-deficient muscle. The opposite approach however, the breeding of a double mutant with dystrophin deficiency and increased electrical and mechanical muscle activity due to Cl\(^-\) channel deficiency (adr mutation; Fig. 3), did not lead to the expected opposite result. The muscle fibers of the adr-mdx mice, although exposed to myotonic discharges, appeared to be more resistant to dystrophin deficiency than those of mdx mice. In was suggested that the observed fiber type transformation to an oxidative phenotype, which is characteristic for myotonic mouse muscle (see sect. ivD), has a protective effect on dystrophin-deficient muscle (189, 270).

9. Summary

We conclude that the dystrophinopathies are disorders of mechanical stability of the sarcolemma. The influx of Ca\(^{2+}\) through mechanically induced membrane lesions is most likely an important pathogenic step in the process of muscle dystrophy. The excess of cytoplasmatic Ca\(^{2+}\) inevitably disturbs the flow of Ca\(^{2+}\) within the calcium cycle (see sect. ivA) and also disturbs, modulating the function of other Ca\(^{2+}\)-binding proteins, important signaling pathways of the muscle fiber (see sect. ivE). Finally, the combination of diverse cellular dysfunctions causes fiber degeneration and muscle dystrophy.

VI. CONCLUDING REMARKS

As shown in this review, Ca\(^{2+}\) has many important and essential functions for skeletal muscle performance. Therefore, any alteration in Ca\(^{2+}\) handling can disturb muscular function. It is evident that Ca\(^{2+}\) not only directly triggers muscle contraction but is also involved in relaxation after the twitch, regulation of energy metabolism, and maintenance of structural integrity of the muscle fiber. Additionally, this ion has long-term effects in that it regulates the transcription of several genes in the nucleus, which obviously has implications in development and differentiation. Because of its complexity, it is largely unknown how all the Ca\(^{2+}\) actions in a single muscle fiber are orchestrated. However, during the recent years, many important structural and functional insights have been gained, and these new developments allowed mechanistic predictions to be made in several instances.

It is evident that a large muscle fiber type diversity and plasticity has evolved. Interestingly, the genetic pro-
gram of a given muscle can be influenced largely by external factors such as neuronal or hormonal activity, exercise, injury, and many other factors, allowing very fine adaptation to special needs and tasks of specialized muscles. Much is known also about the molecular diversity of individual muscle proteins. It seems that specific functions of muscle proteins can be achieved through the following mechanisms: 1) protein isoforms encoded by different genes and combinations thereof; 2) differential transcriptional and posttranscriptional regulation to yield different levels of gene products; and 3) alternative splicing to yield an additional repertoire in diversity.

Although knowledge of this structural diversity is continuously being accumulated, the functional significance of the protein variants is in many cases far from being elucidated. In addition, the signal transduction mechanisms leading to diversity and enabling adaptation depending on external factors are largely unknown. Especially the factors governing fiber type specificity and isoform expression as a result of neuronal or hormonal activity have not been identified so far. The “general” transcription factors such as MyoD, myogenin, and MRF4 or the negative transcription factor Id-1 might be players in a complex regulatory network.

The recent progress in elucidation of genetic diseases by the powerful “reverse genetics” approaches has in several instances directly led to the discovery of important sequences in muscular proteins and given clues to their functions. Prominent examples commented in this review article are, for example, mutations in the RyR, SERCA, and ion channels. In addition to benefits for the progress of muscle physiology, this also gives hope for patients suffering from muscle diseases. For several muscle diseases new diagnostic and therapeutic possibilities have emerged due to the molecular biological findings of the recent years.

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REFERENCES


25. BERCHTOLD MW. Barvlarbin. In: Guidebook to the Calcium...


101. EDWARDS H AND MOSS SE. Annexin VI. In: \textit{Guidebook to the...


182. HOFFMAN EP and GOROSPE JR. The animal models of Duch-


217. HUCHANT-CADIOU C, BONNET V, MEME W, AND LEOTY C. Hypo-


369. NEGELE JC, DOTSON DG, LIU W, LEE SWEENEY H, AND PUTKEY...


442. VITADELLO M, SCHIAFFINO MV, PICARD A, SCARPA M, and