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Review

New aspects of calcium signaling in skeletal muscle cells: implications in Duchenne muscular dystrophy

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Abstract

Calcium is the most ubiquitous second messenger. Its concentration inside the cell is tightly regulated by a series of mechanisms, among which some have been extensively studied in nonmuscle cells. This is the case of the ''store-operated entry of Ca^{2+}'', the uptake of Ca^{2+} by mitochondria and the inositol 1,4,5-trisphosphate (IP_3) cascade. These processes were recently found to be also present in skeletal muscle and are reviewed here. The ''store-operated entry of Ca^{2+}'' allows the refilling of the stores after muscle fiber depolarization and is activated even after a partial depletion of the sarcoplasmic reticulum (SR). The uptake of Ca^{2+} by mitochondria accelerates muscle relaxation and allows the adaptation of ATP supply to the increased energy demand. IP_3 receptors are found in the nuclear envelope and are involved in Ca^{2+} waves propagating from one nucleus to another. This pathway is possibly involved in gene expression regulation. Finally, cytosolic Ca^{2+} buffers like parvalbumins modify $[Ca^{2+}]_i$ transients and, therefore, muscle mechanics.

The importance of these regulation mechanisms is also evaluated in Duchenne muscular dystrophy (DMD), a disease in which impairment of $[Ca^{2+}]_i$ homeostasis has been postulated but remains, however, controversial. This genetic disease is indeed characterized by the absence of a cytoskeletal protein called dystrophin, a situation leading to a disorganization of the cytoskeleton and to an abnormal influx of Ca^{2+}. How this increased entry of Ca^{2+} affects the local concentration of Ca^{2+} in subcellular compartments and whether this process is involved in the development of the disease are still unclear.

Keywords: Inositol 1,4,5-trisphosphate; Mitochondrion; Sarcoplasmic reticulum; Capacitative entry; Parvalbumin; Calcium channel; Transient receptor potential

1. Introduction

The importance of Ca^{2+} in cardiac muscle contraction was discovered in the end of the 19th century [1]. This led to the concept of second messenger and was studied in great detail in cardiac and skeletal muscle [2,3]. The coupling between the stimulation of a skeletal muscle cell by its motoneuron and the contractile response occurs through a unique mechanism, called excitation contraction (EC) coupling, in which the depolarization of the plasma membrane triggers the release of Ca^{2+} from the sarcoplasmic reticulum (SR). Ca^{2+} binds to troponin C and removes the steric inhibition of acto-myosin ATPase by troponin and tropomyosin. Ryanodine receptors (RyRs), the Ca^{2+}-releasing channels of the SR, are opened via a direct physical interaction with the dihydropyridine receptors (DHPRs) which constitute voltage sensors [4] localized in the T-tubules in front of the terminal cisternae of the SR [5]. This mechanism ensures a synchronized response from all the Ca^{2+}-releasing elements. The release of Ca^{2+} can also occur through one or only a few RyRs and induce an increase of $[Ca^{2+}]_i$ in very localized microdomains. These events, called sparks, seem to be controlled either by the DHPRs (probably for type 1 RyRs) or by Ca^{2+} itself (Ca^{2+}-induced Ca^{2+} release, CICR; probably for the type 3 RyRs). At the end of stimulation, Ca^{2+} is pumped back into the SR by sarco/endoplasmic reticulum Ca^{2+} ATPases (SERCA) and muscle relaxes. All these matters have been treated in a number of excellent reviews [5–8] and will not be further detailed here.

It is now unanimously recognized that Ca^{2+} plays a signaling role and controls numerous different functions in all living cells [9]. Different mechanisms involved in the precise control of cytosolic concentration of free calcium ($[Ca^{2+}]_i$) were thus discovered in nonmuscle cells. Such mechanisms like the ''store-operated entry of Ca^{2+}'', the inositol 1,4,5-trisphosphate (IP_3) cascade or the uptake of Ca^{2+} by mitochondria were found ubiquitously but were

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thought not to be important in skeletal muscle signaling probably because their rate was much slower than EC coupling [10] or, in the case of mitochondria, because their affinity for Ca^{2+} seemed much too low. Surprisingly, it is therefore quite recent that these mechanisms were shown to play a role also in skeletal muscle. This review will be focused on these processes. At the end of each paragraph, special attention will be given to the Duchenne muscular dystrophy (DMD) (introduced hereafter), a pathology possibly related to an alteration of $[Ca^{2+}]_i$ homeostasis.

DMD is a progressive neuromuscular disease affecting 1/3500 male birth (reviewed in Refs. [11,12]). It is characterized by an important muscle degeneration (foci of necrotic fibers) followed, at early stages, by an intense regeneration process. As the regenerative capacity diminishes, muscle tissue is progressively replaced by adipose and fibrous connective tissue, leading to an important muscle weakness. This pathology is due to a deficiency in dystrophin, a 427-kDa protein located at the cytoplasmic face of the sarcolemma. In normal skeletal muscle cells, dystrophin is associated at its carboxy-terminal domain, with a complex of transmembrane proteins, called dystrophin-associated-proteins (DAP), that also bind merosin (laminin-2), a component of the extracellular matrix. At its amino-terminal domain, dystrophin binds cytoskeletal F-actin filaments (i.e. non-sarcomeric actin). Dystrophin thus constitutes a link between the cytoskeleton and the extracellular matrix. It is now clear that the alteration of dystrophin expression affects DAPs' targeting to the membrane and causes a disruption of the link between the cytoskeleton and the extracellular matrix. This process has two consequences: (i) the cell membrane is more fragile and can be mechanically damaged during eccentric muscle contraction; (ii) membrane proteins, especially mechanosensitive ion channels, are disregulated (see below). It has been proposed that an alteration of the homeostasis of intracellular calcium might be responsible of muscle degeneration in DMD. This was based firstly on the fact that the total calcium (free and bound calcium) content is doubled in *mdx* mice (a murine model of DMD) muscle and, secondly, that the influx of calcium through the cellular membrane is increased. This led to the hypothesis that dystrophin-lacking fibers suffer from a chronic Ca^{2+} overload, resulting in activation of Ca^{2+}-dependent proteases [13,14].

2. Calcium fluxes through the plasma membrane

2.1. Calcium channels

Patch clamp studies have revealed the presence of stretch-sensitive, voltage-independent Ca^{2+} channels in the plasma membrane of collagenase-isolated muscle fibers [15]. These channels had a conductance of 8 pS (in the presence of 110 mM $[Ca^{2+}]$ in the pipette) and had a poor selectivity, allowing fluxes of Ca^{2+}, Ba^{2+}, Mn^{2+}, Na^{+} and K^{+} [16]. Their activity was increased in dystrophin-deficient fibers, suggesting a regulation by the cytoskeleton. Accordingly, Mn^{2+} influx (measured by Mn^{2+} quenching of fura-2 and indicative of Ca^{2+} influx) was almost doubled in *mdx* fibers in comparison with controls [16,17]. However, the bulk value of $[Ca^{2+}]_i$ stayed in a normal range in *mdx* fibers and in fibers in which Ca^{2+} influx was artificially elevated up to 10 times, suggesting that muscle fibers have very efficient mechanisms of Ca^{2+} extrusion. As expected, in such case of accelerated influx and efflux, $[Ca^{2+}]$ in the submembranous compartment was increased [18,19]. We recently studied fibers maintained in culture for 6 days (d0 to d5) and observed that the open probability of Ca^{2+} channels and the influx of Mn^{2+} peaked at d1 and then declined with time. This process occurred in both normal and *mdx* fibres but was accentuated and very variable in the latter (20% of *mdx* fibers had an influx 10 to 30 times higher than in control). In spite of such influx, 90% of the *mdx* and control fibers kept a normal $[Ca^{2+}]_i$, again suggesting a very robust Ca^{2+} homeostasis. However, we observed an important decrease in the survival of *mdx* fibers (about one quarter) between d1 and d2, which could be reduced by lowering extracellular $[Ca^{2+}]$ [16]. Thus, it seemed that in a fraction of *mdx* fibers population, Ca^{2+} homeostasis was eventually compromised and cells did not survive. In these cells, calpains or other proteases might be activated (possibly locally in the submembranous space), as proposed by Turner et al. [13] (reviewed in Ref. [20]).

2.2. Store-dependent entry of calcium

In most nonexcitable cells, agonist stimulation leads to a biphasic Ca^{2+} signal in the cytosol consisting in a transient increase due to the release of Ca^{2+} from the IP_3-sensitive endoplasmic reticulum followed by a sustained phase due to the entry of Ca^{2+} from the external medium. This secondary influx of Ca^{2+} is stimulated by the depletion of Ca^{2+} intracellular stores of and has been called "capacitative entry" [21] (reviewed in Refs. [22,23]; Fig. 1). The nature of the messenger (CIF, calcium influx factor) carrying the information about the stores content to the activation of a membrane channel (CRAC, Ca^{2+}-release activated Ca^{2+} channel) remains elusive. It might be a diffusible factor [24] like a poly-unsaturated fatty acid [25,26] (reviewed in Ref. [27]) and/or a structural link between the plasma membrane and the stores (conformational coupling between IP_3 receptors and Ca^{2+} channels in the plasma membrane) [28,29]. These mechanisms could additionally involve an exocytosis-like process (reviewed in Ref. [30]). The identification of the channel involved in store-dependent Ca^{2+} entry has been largely investigated in nonexcitable cells. Candidate molecules for this pathway probably belong to the transient receptor potential channel (TRPC) protein family (reviewed in Ref [31]). This has been shown by heterologous expression of TRPC proteins. However, such overexpressed channels usually have a conductance, a selectivity and/or a mode

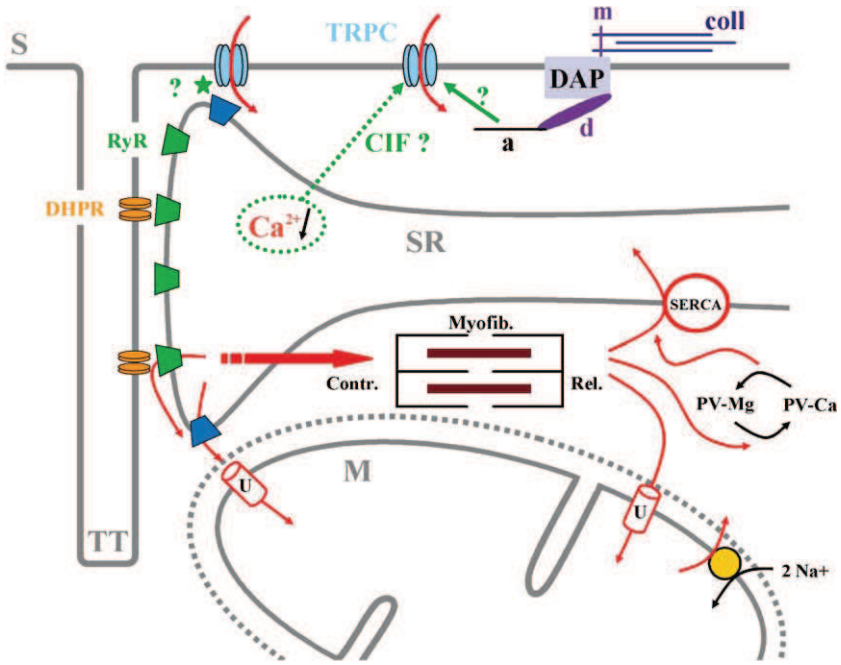

Fig. 1. Ca^{2+} signaling pathways in skeletal muscle cells. Abbreviations—a: actin; SERCA: sarco/endoplasmic reticulum Ca^{2+} ATPase; CIF: calcium influx factor; coll: collagen; Contr.: contraction; d: dystrophin; DAP: dystrophin associated proteins; DHPR: dihydropyrine receptor; M: mitochondria; m: merosin; Myofib.: myofibrils; PV: parvalbumin; Relax: relaxation; RyR: ryanodine receptor; S: sarcolemma; SR: sarcoplasmic reticulum; TRPC: transient receptor potential channel; TT: T-tubule; U: uniporter. Color and form codes—red arrows: Ca^{2+} movements; yellow circle Na^{+}/Ca^{2+} exchanger; green trapezoids: RyRs involved in the EC coupling (present in the junctional SR); blue trapezoids: RyRs (possibly type 3) in the parajunctional SR possibly involved in close contacts between the SR and the mitochondria. "Ca^{2+} store-depletion Ca^{2+} entry coupling" might require a physical link of RyRs with TRPCs (green star) or a diffusible CIF (green dotted arrow). TRPCs might also be regulated by the cytoskeleton (green arrow).

of activation that is/are different from the native store-activated channels. The functional role of TRPC proteins was also evaluated by selectively repressing their expression by transfection with antisense nucleotide sequences. This strategy demonstrated that TRPC4 is part of the native "Ca^{2+}-release activated Ca^{2+} channel" (CRAC) in adrenal cells [32], that TRPC1 and/or TRPC3 are involved in the thapsigargin-induced entry of Ca^{2+} in HEK-293 cells [33], and that TRPC2 protein is involved in store-operated influx of Ca^{2+} in fibroblasts [34].

Store-dependent entry of Ca^{2+} has now also been found in excitable cells such as smooth muscle cells [35] and, recently, Kurebayashi and Ogawa [36] demonstrated the presence of such an influx in adult skeletal muscle fibers. This was unexpected because muscle fibers do not seem to exchange much Ca^{2+} with the extracellular medium. Indeed, they have large amounts of SR rich in Ca^{2+}-pumps (SERCA) and containing a high buffering capacity (calsequestrin and calreticulin). On the other hand, Ca^{2+} extrusion through the plasma membrane is very slow. So almost all of the Ca^{2+} released during contraction are immediately restored after stimulation; accordingly, twitch contractions can thus be produced repeatedly for hours in the absence of extracellular Ca^{2+}. Nevertheless, the authors showed that the store-dependent influx was activated even by a partial depletion of the stores and should therefore be functional in physiological conditions. The influx of Ca^{2+} was sensitive to Ni^{2+}, but resistant to nifedipine and was suppressed by plasma membrane depolarisation. The coupling mechanism between the depletion of the stores and the entry of Ca^{2+} is not known. As mentioned above, several studies strongly suggest the involvement of the IP_3 receptors in capacitative Ca^{2+} entry and skeletal muscle contains low amounts of IP_3 receptors (see below), suggesting that such a coupling is possible. However, it is clear that, in skeletal muscle, SR releases Ca^{2+} essentially through RyRs. Moreover, Kiselyov et al. [37] demonstrated a possible conformational coupling between RyRs and TRPC3, strengthening the idea that store-operated channels might be controlled by RyRs. Accordingly, Pan et al. [38] recently demonstrated that store-dependent entry of Ca^{2+} induced by repetitive electrical stimulations of myotubes was impaired in the absence of RyRs, and when the integrity of the triads was altered by the absence of mitsugumin 29, a synaptophysin-family related protein. The channel involved was sensitive to 2-aminoethoxydiphenyl borate (2-APB) and 1-(beta-[3-(4-methoxy-phenyl)propoxy]-4-methoxyphenethyl)-1*H*-imidazole hydrochloride (SKF-96365), two drugs previously used to inhibit store-dependent channels in nonexcitable cells, but not to verapamil. At variance with Kurebayashi's and Pan's studies, Islam et al. [39] showed that store-dependent entry of Ca^{2+} in L6 myotubes was inhibited by diltiazem and

proposed a model in which Ca^{2+} goes directly from the extracellular medium into the SR by passing through a combined channel formed from the DHPR and the RyR.

In a recent work [40], we identified the molecular nature of the capacitative entry channels in adult skeletal muscle fibers. We first showed that voltage-independent Ca^{2+} channels found in C57 and *mdx* adult fibers (see previous paragraph) were store-dependent, and therefore hypothesized that they might be constituted of TRPC proteins. By using a conserved coding sequence in the different TRPC genes, we then repressed the expression of TRPC1 and TRPC4 by transfection with antisense oligonucleotides and showed that the occurrence of channel activity decreased by a factor of 10. We therefore concluded that the abnormal influx of Ca^{2+} observed in adult *mdx* fibers occurs through store-dependent Ca^{2+} channels constituted, at least partially, of TRPC proteins. The lack of dystrophin and the consequent disorganization of the cytoskeleton observed in dystrophic fibers might therefore induce a disregulation of these proteins, and lead to an increased influx of Ca^{2+}.

3. Sarcoplasmic reticulum

In nonexcitable cells, the major Ca^{2+} signaling pathway involves the phosphoinositide production [41,42]. Skeletal muscle possesses the biochemical machinery able to produce and degrade IP_3 but it is clear that IP_3 is not involved in EC coupling [43], and it is only recently that the functional importance of the IP_3 pathway was established [44]. The nonneuronal form of type 1 IP_3 receptor was found in skeletal muscle, predominantly in oxidative fibers [45]. At a subcellular level in myotubes, these receptors were found in the I-band region of the SR, a region of the SR that is also rich in Ca^{2+} ATPases, and in the nuclear envelope [46]. Interestingly, cell depolarization induced an increase of IP_3 mass. This effect was still visible in the absence of Ca^{2+} entry or Ca^{2+} release but was inhibited by nifedipine, therefore suggesting the involvement of DHPRs. Depolarization could exert its effects by modifying the rate of inositide lipid phosphorylation [44]. In these myotubes, depolarization induced a rapid and global increase of $[Ca^{2+}]_i$ associated with contraction and a much slower and delayed Ca^{2+} wave propagating from one nucleus to another. This slow signal possibly had long term effects. Indeed, within minutes following depolarization, extracellular signal-regulated kinases 1 and 2 (ERKs 1 and 2) and cAMP response element-binding protein (CREB) were phosphorylated, suggesting that this stimulus could regulate gene expression [46].

Cyclic ADP-ribose (cADPR) and nicotinic acid adenine dinucleotide phosphate (NAADP) are two other potent Ca^{2+} mobilizing molecules present in many tissues and acting on RyRs and on unidentified receptors respectively [47,48] (reviewed in Ref. [49]). In skeletal muscle, cADPR potentiated the depolarization-induced Ca^{2+} release but did not trigger Ca^{2+} release by itself [50]. According to Copello et al. [51], muscle RyRs would not directly interact with cADPR or with NAADP but this is still controversial. Obviously more investigation will be necessary to understand the role of these messengers in skeletal muscle.

It is not yet clear to what extent SR is involved in the abnormal Ca^{2+} homeostasis observed in dystrophic muscle. The expression of α1-, α2- and β-subunits of the DHPRs were similar in control and in mdx microsomes. The type 1 RyR and the fast isoform of Ca^{2+} ATPase (SERCA1) were also found to exist in comparable amounts in control and dystrophin-deficient muscles [52]. However, Kargacin and Kargacin [53] found that the SERCA pump was functionally altered (V_{max} reduced by 40%, but normal K_m) but this result was in contradiction with Takagi et al.'s [54] study on skinned fibers reporting an increased leakage of Ca^{2+} from the SR in *mdx* but a normal Ca^{2+} ATPase activity. The concentration of free Ca^{2+} in the SR ($[Ca^{2+}]_{SR}$) was found to be elevated in *mdx* myotubes [19]. This resulted in a higher depolarization-induced release of Ca^{2+}. However, this effect was not found in human DMD myotubes [55]. Interestingly, Culligan et al. [52] also observed a reduction of calsequestrin-like proteins (intraluminal Ca^{2+} buffers), and a reduced Ca^{2+} binding capacity in dystrophic muscles. Finally, the IP_3 signaling pathway might also be affected since both the level of IP_3 (at rest or after depolarization) and the amount of IP_3 receptors were significantly increased in *mdx* myotubes [56]. This could alter gene expression activity and might therefore be important in the pathophysiology of the disease.

4. Mitochondria

It has been known for a long time that mitochondria possessed the necessary machinery to transport Ca^{2+} into and out of their matrix: Ca^{2+} entry is promoted by a very large electrochemical gradient and occurs through a ruthenium red sensitive uniporter; Ca^{2+} efflux occurs via an electroneutral exchange with 2 Na^+ or 2 H^+ ions. Initially, these processes were proposed to contribute to $[Ca^{2+}]_i$ homeostasis. In the 1980s, it was also suggested that Ca^{2+} ions might play a role in the regulation of mitochondrial metabolism. This was based on the fact that pyruvate dehydrogenase, 2-oxoglutarate dehydrogenase and NAD^+-isocitrate dehydrogenase, three key enzymes involved in NADH formation (and hence ATP synthesis) were activated by Ca^{2+} in the range 0.1 to 10 μM (see Ref. [57] for review). This mechanism explained how a cell could provide ATP supply in response to an increased demand without any decrease in the ATP/ADP ratio. However, for a long time, the physiological relevance of these observations remained controversial because of the very low affinity of the uniporter for Ca^{2+} (half-maximal rate of Ca^{2+} transport observed for $[Ca^{2+}]$ levels above 30 μM and very low uptake for $[Ca^{2+}]$ below 10 μM [58]). Opinion changed

when, in 1992, Rizzuto et al. [59] developed the strategy of targeted recombinant aequorin allowing to follow Ca^{2+} concentration in subcellular compartments, e.g. in mitochondria ($[Ca^{2+}]_{mit}$) and provided a clear demonstration that agonist-induced $[Ca^{2+}]_i$ elevations evoke rapid and transient increases of $[Ca^{2+}]_{mit}$. The activation of such Ca^{2+} uptake in mitochondria resulted from the high $[Ca^{2+}]_i$ reached in very localized microdomains close to the IP_3 or RyRs [60] (up to 20–40 μM [61]; Fig. 1); these Ca^{2+} microdomains could be sensed by mitochondria because of their close apposition to the ER [62]. So it became more and more evident that endoplasmic reticulum and mitochondria form structural and functional units that allow mitochondria to sense $[Ca^{2+}]_i$ oscillations more than slow and global increases of $[Ca^{2+}]_i$, the frequency of these oscillations controlling the activation of calcium-sensitive dehydrogenases [63]. But it is now also clear that mitochondria play a role in $[Ca^{2+}]_i$ homeostasis. In sympathetic neurons, for example, they diminish $[Ca^{2+}]_i$ transients induced by fast depolarization (due to the accumulation of calcium from the cytosol) and slow down the recovery phase (release of accumulated Ca^{2+}) [64]. In other cases, mitochondria can directly recycle Ca^{2+} back to the reticulum, reducing the store depletion during agonist stimulation [65].

The situation seems particular in striated muscle, since RyRs are located in specialized regions of the SR (junctional SR) which face the T-tubules and form the so-called triads [5]. In the triads, DHPRs of the T-tubule plasma membrane are physically connected to the RyRs (probably RyR1) of the SR in a very precise stoichiometry (4 DHPRs attached to every other RyR). It is therefore probable that Ca^{2+} is released from SR regions essentially devoid of mitochondria. This picture is, however, too simple: DHPR/RyR ratio measured in various muscles is usually lower than 2, suggesting that a nonnegligible proportion (e.g. 30–40% in rat soleus [66]) of RyRs are present in nonjunctional regions of the SR and could therefore be involved in CICR. Felder and Franzini-Arsmstrong [67] have recently shown that RyR3 were localized in a parajunctional position to the RyR1. These parajunctional regions could therefore also form close contacts with mitochondria as it has been proposed in the case of cardiac muscle [68] (Fig. 1). In any case, $[Ca^{2+}]_i$ transients are much larger in skeletal muscle than in any other cells, reaching 3 to 7 μM [69,70]. Moreover, the K_m for Ca^{2+} of the uniporter was around 1 and 2.5 μM in mitochondria isolated from fast and slow skeletal muscles, respectively (determined in physiological conditions of $[Mg^{2+}]$), i.e. a value lower than in other tissues (see above) [71]. Mitochondria might thus be able to sense high $[Ca^{2+}]_i$ and participate in Ca^{2+} homeostasis also in skeletal muscle. The role of such participation was indeed demonstrated in mechanically skinned fibers [72]: ruthenium red prolonged the contractile activity (presumably due to Ca accumulation in the cytoplasm) and slowed down relaxation. This effect was observed in type I and IIA fibres (slow, oxidative fibres rich in mitochondria) but absent in type IIB fibres (fast glycolytic fibres, poor in mitochondria), suggesting that it was due to the inhibition of the mitochondrial uniporter by ruthenium red and not to its potential inhibitory effects on RyRs [73]. Note that the role of mitochondria in the recovery phase may appear opposite to what is observed in neurons (see above [64]): in neurons, during the recovery phase, mitochondria release the previously accumulated Ca^{2+}, thus slowing down the decay of $[Ca^{2+}]_i$ whereas in skeletal fibers, mitochondria accumulate Ca^{2+} during the recovery phase and therefore accelerate relaxation. This difference is probably due to the very fast transients observed in skeletal muscles and/or to the different structural disposition of SR and mitochondria. It has to be assumed that, at the end of relaxation, Ca^{2+} accumulated in mitochondria is restored into the SR without $[Ca^{2+}]_i$ increase (no contraction). This might be rendered possible by the proximity of mitochondria with nonjunctional SR rich in SERCA pumps (Fig. 1).

Is $[Ca^{2+}]_{mit}$ regulation normal in dystrophic muscle? Robert et al. [19] recently showed that in normal myotubes in culture, depolarization induced a peak of $[Ca^{2+}]$ in the cytosol that was sensed and amplified in mitochondria ($[Ca^{2+}]_{mit}$ peak around 10 μM). This response was increased in *mdx* myotubes ($[Ca^{2+}]_{mit}$ peak around 15–20 μM). Such increased Ca^{2+} accumulation in mitochondria might trigger fiber apoptosis which seems to precede necrosis in dystrophic muscle [74,75].

5. Cytosolic calcium buffers

Fast-contracting skeletal muscles from lower vertebrates and from small mammals contain high amounts of parvalbumin (0.5 to 1 mM), a cytosolic Ca^{2+} buffer. Parvalbumin has two high affinity binding sites for Ca^{2+} ($K_a = 10^8\ M^{-1}$). These sites can also bind Mg^{2+} with a 10^4 lower affinity. In the resting state ($[Mg^{2+}] = 1$ to 3 mM and $[Ca^{2+}]_i = 20$ to 50 nM), parvalbumin therefore mainly binds Mg^{2+} [76]. During stimulation, when $[Ca^{2+}]_i$ increases, Ca^{2+} binds to troponin C in spite of the fact that its affinity for Ca^{2+} is lower than parvabumin affinity for Ca^{2+}. This is due to the fact that Ca^{2+} binding rate to parvalbumin is limited by the much slower dissociation of Mg^{2+}, whereas Ca^{2+} binding to troponin C (which does not bind Mg^{2+} in physiological conditions) is only limited by the Ca^{2+} diffusion. At the end of stimulation, parvalbumin is able to store the Ca^{2+} previously bound to troponin C and can therefore accelerate relaxation (for review see Ref. [77]). Ca^{2+} is then progressively removed from the Ca–parvalbumin complex and pumped into the SR by the Ca^{2+} ATPase (which has an even greater affinity for Ca^{2+} than parvalbumin; see Fig. 1). As the binding capacity of parvalbumin is limited, the rate of relaxation slows exponentially with increasing contraction duration (due to the saturation of parvalbumin) [78–80]. This saturation of parvalbumin and the consequent

slowing of relaxation rate were limited by Mg^{2+} dissociation. Recently, we investigated this process in a mouse strain where the parvalbumin gene had been knocked-out [81]. As expected, in the normal mouse, we observed that in two fast muscles, namely extensor digitorum longus (EDL) and flexor digitorum brevis (FDB), increased tetanus duration reduced the rate of relaxation until an approximately constant value was obtained. The tetanus duration necessary to eliminate the contribution of parvalbumin to relaxation was lower in FDB than in EDL, in agreement with the fact that the average parvalbumin content in FDB muscle was also lower than in EDL. In parvalbumin-deficient mice, muscle relaxation was slow and did not depend on tetanus duration. More interestingly, the comparison between normal and parvalbumin-deficient muscles revealed a property that was not expected from the 'saturation' model presented so far. Indeed, at all tetanus durations, relaxations were slower in parvalbumin-deficient muscles than in normal, (i.e. even after long tetani when parvalbumin is supposed to be Ca-saturated). This property is reminiscent of the 'shuttle' model proposed a long time ago [82]. This model, inspired by the role of myoglobin in oxygen transport, suggests that parvalbumin facilitates Ca^{2+} diffusion from the myofibrils to the SR, a property that has been established in vitro [83]. Besides, it is interesting to note that parvalbumin interacts with the SR and stimulates Ca^{2+} uptake [84], an effect that could enhance this shuttle effect on relaxation.

We recently investigated the possible 'protective' role of parvalbumin in the development of mouse Duchenne-type dystrophy. The reasons were the following: (i) The very benign manifestations of the dystrophy in mice sharply contrast with the severe and life-threatening evolution in humans. (ii) Dystrophin-lacking fibers suffer from an increased Ca^{2+} influx, resulting in activation of Ca^{2+}-dependent proteases. However, several laboratories have consistently reported that basal cytosolic Ca^{2+} levels were identical in *mdx* and wild-type mice (reviewed in Ref. [12]), suggesting that a compensatory mechanism could be present. We indeed observed increased mRNA levels of the intracellular calcium buffer parvalbumin [85]. (iii) In humans, Duchenne dystrophy predominantly affects type IIB muscle fibers which are progressively replaced by oxidative type I and IIA fibers [86]. This phenomenon, though present, is much less pronounced in muscles from *mdx* mice. Parvalbumin is found in large amounts in these fibers in mice but undetectable in humans [87].

In order to test the hypothesis that, in muscle from *mdx* mice, parvalbumin contributed to maintain a mild dystrophic phenotype, possibly by preventing Ca^{2+}-dependent activation of proteases, we generated double mutant mice lacking both dystrophin and parvalbumin [88]. Compared to the *mdx* phenotype, these double knock-out animals presented a more important muscle pseudo-hypertrophy, an accentuated process of degeneration and regeneration and an increased fibrosis in late adulthood. This suggests that Ca^{2+} transients (that are affected by parvalbumin), more than resting $[Ca^{2+}]_i$ (which is not affected), have an influence on the development of pathological processes. These transients could be sensed by calpains, which have been recently shown to be more activated by presenting Ca^{2+} in a pulsatile manner than by a steady-state $[Ca^{2+}]$ corresponding to the time-average of transients [89]. Mitochondria (see above) constitute other Ca^{2+} oscillations "detectors" possibly involved in the disease.

6. Conclusions

There are now evidences that a series of mechanisms regulate $[Ca^{2+}]_i$ in skeletal muscle cells in addition to the well characterized EC coupling. The study of these mechanisms is crucial to understand pathological processes involved in muscle diseases such as DMD. This also means that skeletal muscle, the tissue in which the notion the second messenger was established several decades ago, stays an interesting model whose special differentiation and architecture can reveal new aspects of $[Ca^{2+}]_i$ signaling.

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