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REVIEW

The excitation–contraction coupling mechanism in skeletal muscle

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Abstract First coined by Alexander Sandow in 1952, the term excitation–contraction coupling (ECC) describes the rapid communication between electrical events occurring in the plasma membrane of skeletal muscle fibres and Ca^{2+} release from the SR, which leads to contraction. The sequence of events in twitch skeletal muscle involves: (1) initiation and propagation of an action potential along the plasma membrane, (2) spread of the potential throughout the transverse tubule system (T-tubule system), (3) dihydropyridine receptors (DHPR)-mediated detection of changes in membrane potential, (4) allosteric interaction between DHPR and sarcoplasmic reticulum (SR) ryanodine receptors (RyR), (5) release of Ca^{2+} from the SR and transient increase of Ca^{2+} concentration in the myoplasm, (6) activation of the myoplasmic Ca^{2+} buffering system and the contractile apparatus, followed by (7) Ca^{2+} disappearance from the myoplasm mediated mainly by its reuptake by the SR through the SR Ca^{2+} adenosine triphosphatase (SERCA), and under several conditions movement to the mitochondria and extrusion by the Na^{+}/Ca^{2+} exchanger (NCX). In this text, we review the basics of ECC in skeletal muscle and the techniques used to study it. Moreover, we highlight some recent advances and point out gaps in knowledge on particular issues related to ECC such as (1) DHPR-RyR molecular interaction, (2) differences regarding fibre types, (3) its alteration during muscle fatigue, (4) the role of mitochondria and store-operated Ca^{2+} entry in the general ECC sequence, (5) contractile potentiators, and (6) Ca^{2+} sparks.

Keywords Excitation–contraction coupling · Ca^{2+} transients · Skeletal muscle · Fibre types · Mitochondria

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Introduction

The excitation–contraction coupling (ECC) phenomenon was defined by Alexander Sandow as the series of events occurring from the generation of the action potential (AP) in the skeletal muscle fibres to the beginning of muscle tension (Kahn and Sandow 1950; Sandow 1952). It has been more than 60 years since his early work on skeletal muscle, during which the temporal and spatial resolution of the techniques to study ECC have greatly improved, reaching a capacity for discrimination at a molecular level. Since then, a great amount of information on ECC morphological basis, physiological importance, and pharmacological modulation, initially in amphibians and more recently in mammalians, has been gathered.

Here, we review the basics of ECC, the techniques used to understand the phenomenon and the most recent advances in ECC knowledge, focused on the information gathered using fast Ca^{2+} dyes in mammalian preparations and on important issues still under research. These issues include the nature of the interaction among key molecules in ECC, the regulation of the ECC mechanism in different skeletal muscle fibre types, its role in phenomena such as fatigue, its drug modulation, the store-operated Ca^{2+} entry (SOCE) –mitochondria–ECC relationship and Ca^{2+} sparks.

The excitation–contraction coupling (ECC) mechanism in skeletal muscle

Ca^{2+} cell homeostasis and signalling result from dynamic interactions between mechanisms that provoke an increase of cytoplasmic free Ca^{2+} and those that reduce it. In the specific case of striated muscles, contraction and relaxation mechanisms are both regulated by rapid changes in myoplasmic free Ca^{2+} concentration. Even before knowing the molecular machinery involved in Ca^{2+} handling, it was shown that Ca^{2+} was the activator of the contractile mechanism (Heilbrunn and Wiercinsky 1947; Niedergerke 1955; Weber 1959), and that its concentration increase in the myoplasm elicited by electrical stimulation, precedes tension generation (Ridgway and Ashley 1967; Ebashi et al. 1969). It was also known that muscle contraction depends on Ca^{2+} released from the SR (Caputo and Giménez 1967; Armstrong et al. 1972) and that, after activation, most of the Ca^{2+} released goes back finally to the SR (Hasselbach and Makinose 1961; Hasselbach 1964; Winegrad 1968).

The ECC phenomenon represents a fast communication between electrical events occurring in the plasma membrane and Ca^{2+} release from the SR, which leads to muscle contraction. The sequence of events in skeletal twitch muscle fibres involves: (1) initiation and propagation of an AP along the plasma membrane, (2) radial spread of the potential along the transverse tubule system (T-tubule system), (3) dihydropyridine receptors (DHPR, L-type Ca^{2+} channel $Ca_V1.1$)-mediated detection of changes in membrane potential, (4) allosteric interaction of the DHPR with the sarcoplasmic reticulum (SR) ryanodine receptors (RyR), (5) release of Ca^{2+} from the SR and transient increase of Ca^{2+} concentration in the myoplasm, (6) transient activation of the myoplasmic Ca^{2+} buffering system and the contractile apparatus, followed by (7) disappearance of Ca^{2+} from the myoplasm mediated by its movement to the mitochondria, its transport by the Na^+/Ca^{2+} exchanger (NCX) and its final reuptake by the SR through the SR Ca^{2+} adenosine triphosphatase (SERCA) (Sandow 1952; Caputo 1983; Fill and Copello 2002; Calderón-Vélez and Figueroa-Gordon 2009).

In twitch skeletal muscle cells, both the differential and selective conductance and the ion distribution across the membrane generate a resting potential of about –85 mV, with the interior of the cell negative compared to the exterior (Horowicz 1961; Luff and Atwood 1972). The acetylcholine neurotransmitter released into the motor plate by the inferior motor neuron acts as an initiator for the AP in muscle fibres causing the transmembrane potential change to reach values of up to 100 mV, through voltage-dependent ionic conductance changes (Horowicz 1961; Hodgkin and Huxley 1952; Luff and Atwood 1972). Since the AP is a regulator of ECC, its modifications (see below) may affect the kinetics of muscle contraction (Hodgkin and Horowicz 1960; Sandow et al. 1965). Experimentally, membrane depolarization can be achieved by replacing neurotransmitters with direct electrical stimulation or by increasing the extracellular K^+ concentration (Hodgkin and Horowicz 1959). Additionally, contractile activation can be induced bypassing the membrane depolarization step, for instance with the help of caffeine (Axelsson and Thesleff 1958; Endo 1975).

The plasma membrane AP propagates longitudinally along the fibre and radially along the T-tubules, electrotonically (Adrian et al. 1969), or in a fast, homogeneous, regenerative Na^+-dependent process (Huxley 1964; González-Serratos 1971; Bezanilla et al. 1972). T-tubules are invaginations of the plasma membrane that transversely extend into muscle fibres (they also have longitudinal components inside the fibre), and serve as a mean to rapidly carry electrical information from the surface to the interior of the muscle fibre, namely to the DHPR facing the SR membrane (Franzini-Armstrong and Porter 1964; Bezanilla et al. 1972; Edwards et al. 2012). The depolarization of the surface reaches the triadic region, where one T-tubule is surrounded by junctional SR segments from two different terminal cisternae (Porter and Palade 1957; Franzini-Armstrong and Porter 1964; Peachey 1965). In this region, arrays of molecules known as RyR and further identified as Ca^{2+} release channels face, vis à vis, but in alternate mode, the DHPR, located in the T-tubule membranes and arranged in groups of four, called tetrads (Block et al. 1988; Franzini-Armstrong and Jorgensen 1994; Franzini-Armstrong 1999). DHPR are heteropentamers formed by subunits α_1, α_2, β, γ, and δ, whose function is regulated by membrane potential; the S4 transmembrane domains of α_1 subunit constitute the voltage sensors. The electrical manifestation of the voltage sensor operation is a non-linear intramembrane charge movement, characterized by S-shaped voltage dependence (Schneider and Chandler 1973; Ríos and Pizarro 1991). The voltage change induces a still unclear conformational change in the DHPR that gates the opening of the RyR in a cooperative manner (see below) (Schneider and Chandler 1973; Ríos and Brum 1987; Ríos and Pizarro 1991; Ríos et al. 1993; Bezanilla 2000). DHPR function and expression is also regulated by the RyR and the JP-45/CSQ complex (Ávila and Dirksen 2000; Treves et al. 2009; Mosca et al. 2013).

RyR are high molecular weight (~550 kDa per monomer) homotetramers whose shape has been classically associated to that of a mushroom: a bulky cytoplasmic domain which has regulatory sites, and a transmembrane domain which allow the protein to function as a large conductance Ca^{2+} channel that regulates Ca^{2+} outflow from the SR to the cytoplasm (see below) (Smith et al. 1988; Lai et al. 1988; Takeshima et al. 1989; Wagenknecht et al. 1989; Franzini-Armstrong and Jorgensen 1994; Franzini-Armstrong 1999; Serysheva et al. 2007). Regions for intraluminal regulation have also been described (Goonasekera et al. 2007). The electron microscopy appearance of the RyR proteins is known as "feet". RyR

activity and expression are regulated by adenosine triphosphate (ATP), Mg^{2+}, Ca^{2+}, redox status, phosphorylation/dephosphorylation status, and several proteins including calsequestrin (CSQ), calmodulin, S100A1, FK 506 binding protein (FKBP), calumenin, triadin, junctin, and possibly by the SR protein-27 (SRP-27) and indirectly by SR protein 35 (SRP-35) (Lai et al. 1988; Meissner 1984; Coronado et al. 1994; Fill and Copello 2002; Wei et al. 2006; Jung et al. 2006; Goonasekera et al. 2007; Bleunven et al. 2008; Prosser et al. 2008; Treves et al. 2009, 2012; Wium et al. 2012).

In skeletal muscle, the RyR1 and the DHPR are in close apposition, constituting the Ca^{2+} release units (CRU), that are activated almost simultaneously as a response to an AP (Franzini-Armstrong and Jorgensen 1994; Franzini-Armstrong 1999). Due to the alternate disposition of RyR and DHPR arrays, for each RyR under control of a tetrad, there is one molecule that is not (Franzini-Armstrong and Jorgensen 1994). In amphibian muscle fibres, the sizeable presence in the parajunctional region of another isoform of the RyR (Felder and Franzini-Armstrong 2002) the RyR3, increases the fractional number of RyR that are not under DHPR control. The alternate arrangements of RyR and DHPR, and the presence of extrajunctional RyR3, constitute the structural basis for the proposal of a dual mechanism of Ca^{2+} release (O'Brien et al. 1995; Ríos and Pizarro 1988). According to this, RyR facing DHPR are under control of the membrane potential while RyR that do not face DHPR are activated by Ca^{2+} through a positive feedback mechanism, initially studied in frog muscles, known as Ca^{2+}-induced Ca^{2+} release (CICR) (Endo et al. 1970; Fabiato 1984; Endo 2009). Then, the RyR activation in the triadic region provokes an outflow of Ca^{2+} into the myoplasm (Smith et al. 1988; Escobar et al. 1994). The CICR mechanism, however, may not participate in the massive Ca^{2+} release in mammalian muscle, as it does in frog muscle (Figueroa et al. 2012), since the voltage-activated Ca^{2+} release mechanism is responsible for the rapid, and highly synchronized, Ca^{2+} release in mammalian muscles.

The rate of Ca^{2+} release from the SR may be over 200 μmoles/ms in fast-twitch mammalian fibres and the cytoplasmic free Ca^{2+} concentration may increase up to 20 μM (Baylor and Hollingworth 2003), from a resting concentration close to 100 nM (Williams et al. 1990). Nonetheless, the peak values reported vary depending on the Ca^{2+} dye used, the calibration technique, the muscle type, whether it is amphibian or mammalian, and the muscle fibre type, slow or fast-twitch (Miledi et al. 1977; Klein et al. 1988; Konishi et al. 1991; Delbono and Stefani 1993; Shirokova et al. 1996; Baylor and Hollingworth 2003; Calderón et al. 2009, 2010, 2013). The amount of Ca^{2+} released during a single twitch in rat fast-twitch fibres seems to be constant over a range of SR Ca^{2+} content and equals about 20 % of the endogenous Ca^{2+} load (Posterino and Lamb 2003). For both slow- and fast-twitch fibres, total SR free Ca^{2+} content ranges from about 0.5 to 1 mM and can hardly be depleted (Fryer and Stephenson 1996; Wang et al. 2012). The high amount of Ca^{2+} inside the SR is buffered by CSQ, whose conformation and affinity changes determines its ability to also regulate RyR and then modulate Ca^{2+} release (Wei et al. 2006; Royer et al. 2010). Once released, the Ca^{2+} spreads in a matter of milliseconds, and interacts with troponin C. Ca^{2+}-troponin interaction eliminates the inhibition imposed by troponin I and tropomyosin on the actin–myosin interaction, allowing the thin filaments to slide over the thick ones, thus producing tension (Huxley 1969; Ebashi 1974; Baylor and Hollingworth 2003; Craig and Padrón 2004).

Cytoplasmic Ca^{2+} removal rate is approximately 50 μmoles/ms and is initially buffered by soluble cytoplasmic proteins such as parvalbumin and is finally removed from the cytosol by the action of the SERCA, the mitochondria and the NCX, with fibre type-dependent kinetics (see "ECC and fibre types") (Hasselbach 1964; Heizmann et al. 1982; Gillis et al. 1982; Balnave and Allen 1998; Baylor and Hollingworth 2003). Cytosolic proteins transiently buffer Ca^{2+} with moderate kinetics, while the SERCA takes the Ca^{2+} back to the SR with slower kinetics. Parvalbumin is a 12 kDa monomeric protein, that binds Ca^{2+} with high affinity and also binds Mg^{2+}, playing a crucial role in fast muscles' relaxation (see below) (Heizmann et al. 1982; Füchtbauer et al. 1991). Mitochondria and NCX are also slow Ca^{2+} handling mechanisms. Recently, Ca^{2+} uptake by mitochondria has been directly visualized in living cells, using genetically encoded chemiluminescent and fluorescent Ca^{2+} sensors, specifically targeted to the mitochondrial matrix (see below) (Rizzuto et al. 1992; Rudolf et al. 2004; Rogers et al. 2007) and NCX has been shown to activate under different conditions (Balnave and Allen 1998; Calderón et al. unpublished results). Most of these mechanisms finally deliver Ca^{2+} to the SERCA. SERCA is a 110 kDa, type P pump, mainly located in the SR's longitudinal region; factors regulating its expression and function include Ca^{2+}, pH, thyroid hormones, and phospholamban and sarcolipin proteins (Odermatt et al. 1998; Hasselbach 1964, 1998; Hasselbach et al. 1975; Jorgensen and Jones 1986; James et al. 1989; Martonosi and Pikula 2003; Periasamy and Kalyanasundaram 2007). Three different genes encode 3 SERCA isoforms, but the number of isoforms produced by alternative splicing is higher (Martonosi and Pikula 2003; Periasamy and Kalyanasundaram 2007). This protein has 3 large cytoplasmic domains attached to a domain consisting of 10 hydrophobic trans-SR-membrane helices. Large movements, mainly of the cytoplasmic domains, mediate the Ca^{2+} pumping from the cytoplasm into the SR against its concentration gradient, reducing the cytoplasmic Ca^{2+} levels to nanomolar values in a matter of milliseconds, thus ensuring a fast relaxation of the skeletal muscles (Hasselbach 1964; MacLennan et al. 1985; Martonosi and Pikula 2003; Toyoshima and Mizutani 2004).

Since some mechanisms (Ca^{2+} pumps, NCX and Ca^{2+} leak) may take Ca^{2+} out of the cells, with a yet not well known kinetics in skeletal muscle, a mechanism to ensure store Ca^{2+} replenishment exists (see below) (Parekh and Penner 1997; Kurebayashi and Ogawa 2001; Pan et al. 2002; Gonzalez-Narvaez and Castillo 2007; Bolaños et al. 2009).

The previous description gives a brief overview of most of the knowledge gathered on ECC coupling in skeletal muscle over the past 60 years, using a combination of experimental approximations, some of which will be discussed below.

Technical issues on the ECC study

Cell preparations used in ECC study

Enzymatic dissociation and hand dissection

Intact muscle fibres for physiological experiments can be obtained by means of enzymatic dissociation and manual isolation. In the first technique, described by Bekoff and Betz (Bekoff and Betz 1977) and modified by others (Caputo et al. 2004; Calderón et al. 2009; Calderón 2013), different rat or mouse muscles (mainly flexor digitorum brevis (FDB), extensor digitorum longus –EDL-, soleus and interossei) are subjected to an enzymatic dissociation with collagenase to digest the connective tissue surrounding the fibres, and subsequently subjected to mechanical dissociation through the use of glass-pipettes. The procedure yields complete, tendon-free muscle fibres. Once obtained, about 85 % of the fibres contract immediately and remain excitable for up to 24-36 hours when kept in Tyrode solution or culture medium (Calderón et al. 2009, 2010; Calderón 2013). One limitation with the use of dissociated fibres is their susceptibility to movement artifacts when recording Ca^{2+} transients. This drawback, however, has been overcome with the use of N-benzyl-p-toluene sulphonamide (BTS), butanedione monoxime (BDM) (Sun et al. 2001) and laminin. BTS is a small molecule which inhibits myosin type II and avoids shortening of the fibres (Cheung et al. 2002; Shaw et al. 2003; Calderón et al. 2009, 2010). Laminin works as a substrate to which muscle fibres adhere, limiting their movement and allowing the recording of movement artifacts-free Ca^{2+} transients with the advantage of working for all fibre types (Calderón et al. 2009, 2010).

Manual isolation appeals to the researcher's ability to dissect a muscle to obtain bundles with usually a few tens of fibres, or to obtain an isolated fibre still attached to its tendons. The fibre's integrity can be visually verified by observing its response to an electrical stimulus (Caputo and Giménez 1967; Lännergren and Westerblad 1987; Baylor and Hollingworth 2003; Bruton et al. 2003).

Although it has been suggested that fibres may be damaged during the enzymatic dissociation procedure (Hollingworth et al. 2012), the morphological evaluation and measurements of the levels of resting basal Ca^{2+}, electrical properties of the sarcolemma, charge movement, amplitude of the AP and release of Ca^{2+} from the SR (Bekoff and Betz 1977; Williams et al. 1990; Szentesi et al. 1997; Woods et al. 2004; Wang et al. 2007) have shown that enzymatic dissociation of different muscles renders functionally intact fibres. Moreover, recent results showed that these fibres reproduce results previously described in manually isolated ones, such as the fatigue-induced increase in intramitochondrial Ca^{2+} and the tetanic Ca^{2+} transient's amplitude reduction (Bruton et al. 2003; Calderón et al. 2011). Discrepancies between findings reported in different works (Calderón et al. 2010; Hollingworth et al. 2012) may have arisen from intrinsic differences between both preparations, such as the reduced sarcomere length found in dissociated fibres compared to manually isolated fibres mounted on transducers (Bolaños et al. 2008; Calderón et al. 2009).

Mechanical and chemical fibre skinning techniques

These techniques allow direct access to the interior of the muscle fibre, as both of them remove the sarcolemma, either mechanically (by microdissection) or with the use of glycerol or soft detergents such as Triton X-100 (Natori 1954; Wood et al. 1975; Lamb et al. 1995; Fryer et al. 1995; Knuth et al. 2006). The SR function can be preserved depending on the intensity of the treatment. It has been proven that in mechanically skinned fibres the T-tubules are resealed forming a closed compartment that allow the re-establishment of ionic gradients and are capable of conducting AP. At the same time, the myoplasmic compartment remains open for experimental manipulation (Lamb et al. 1995; Fryer et al. 1995). One can hence determine, for instance, the effect of a change in pH or concentration of a metabolite like phosphate (Pi) or lactate on the myofibrils sensitivity to Ca^{2+}, and the maximum strength generated with a saturating amount of cytosolic Ca^{2+}. As a disadvantage, these techniques may remove cytosolic compounds like gluthathione, ATP, and parvalbumin, which can make the reading of certain results somewhat difficult.

Cut fibres preparation

This preparation allows control of the fibre membrane potential by double or triple vaseline gap voltage clamp techniques and also gives access to the myoplasm (Hille and Campbell 1976; Kovács and Schneider 1978; Kovács et al. 1983). The fibres, usually from frog or rat, are manually dissected and then cut, keeping or not part of the tendons. After that, the fibres are mounted on a chamber that allows the control of the composition of the intracellular medium through the cut fibre ends while the central portion of the fibre is electrically isolated from the cut extremes, using vaseline strips.

Cell cultures

Primary cultures and well-established myogenic cell lines, such as the mouse (C_2C_{12}) and rat (L6), or the dyspedic 1B5 (Yaffe and Saxel 1977; Rando and Blau 1994; Moore et al. 1998), have ideal characteristics for the in vitro study of differentiation, development, and signalling, on a functional, biochemical, and molecular level. In both cases (primary cultures and cell lines), the cultures must be kept in a growth medium with up to 20 % fetal bovine serum (FBS) until they reach ~60–90 % confluence to subsequently be induced to form myotubes, by reducing the amount of FBS in the culture medium or by its substitution for horse serum between 2 and 5 %. The functional results are limited to comparisons with early in vivo developmental stages, mainly because of the structural characteristics reached by myotubes formed in culture. When fibres isolated by enzymatic dissociation of FDB muscles from adult mice are kept in serum-free culture medium, they retain normal ECC properties for up to 7 days and are suitable for different physiological studies (Wang et al. 2007).

Experimental procedures

Wide-field quantitative fluorescence and measurements of Ca^{2+} transients

Over 45 years ago, Ridgway and Ashley (1967) were able to record global Ca^{2+} transients in electrically stimulated, intact muscle fibres. The authors injected the photoprotein aequorin, which emits light in presence of Ca^{2+}. Due to the technique difficulty and stoichiometric problems, aequorin was substituted by metallochromic dyes, whose absorption spectra shift in the presence of Ca^{2+}. However, these dyes were also substituted by the fluorescent ones due to the unsurpassed experimental advantages of the latter.

Usually, Ca^{2+} transients records obtained using fluorescent molecules have the following kinetic characteristics: (1) a rising phase, which reflects the Ca^{2+} outflow from the SR, and its free presence in the cytoplasm, (2) a peak, when Ca^{2+} outflow stops and Ca^{2+} removal mechanisms are already activated, and (3) a decay phase, which represents the sole operation of myoplasmic free Ca^{2+} removal mechanisms (Fig. 1). Ca^{2+} transients can be obtained in enzymatically dissociated or manually dissected fibres, in myotubes, and in cut fibres (Kovács et al. 1983; Westerblad and Allen 1991; Delbono and Stefani 1993; Caputo et al. 2004; Calderón et al. 2010). For these measurements, fluorescent indicators coupled to an acetoxymethyl (AM) ester moiety are commonly used, which allows them to diffuse into the cell. Once in the cytoplasm, endogenous esterases release the indicator molecule, which is now ready to bind Ca^{2+} and emit light (Tsien 1981). Fluorescent indicators in its salt form can also be injected into the cells, giving the possibility of determining the intracellular dye concentration and other dye-related data in a reliably way (Baylor and Hollingworth 1988; Konishi et al. 1991; Westerblad and Allen 1992).

In general, fluorescent Ca^{2+} dyes can be classified as ratiometric or non-ratiometric (Grynkiewicz et al. 1985; Minta et al. 1989; Raju et al. 1989; Takahashi et al. 1999; Katerinopoulos and Foukaraki 2002; Kao et al. 2010; Bruton et al. 2012). Non-ratiometric dyes can be excited with visible light and, when bound to Ca^{2+}, their fluorescence intensity increases without showing an important maximum excitation or emission wavelength shift. In this group, low and high affinity dyes can be found, having a dissociation constant in the micromolar or nanomolar range, respectively. The most commonly used dyes of this type are Mag-Fura-2 (first described as ratiometric, also giving the possibility of being used as non-ratiometric; Raju et al. 1989; Konishi et al. 1991; Baylor and Hollingworth 2003), Fluo-3 (Caputo et al. 1994), Fluo-4 (Prosser et al. 2010), Rhod-2 (Escobar et al. 1994; Caputo et al. 1999; Bruton et al. 2003) and Mag-Fluo-4 (Caputo et al. 2004; Calderón et al. 2009, 2010). The most suitable dyes to study ECC in skeletal muscle seem to be the low affinity Ca^{2+} dyes, Mag-Fura-2 and Mag-Fluo-4 (Fig. 1), since they are well known and can reliably track fast, large and brief Ca^{2+} transients such as those found in skeletal muscle (Hollingworth et al. 2009; Baylor and Hollingworth 2011; Calderón et al. 2010, 2013). Their disadvantages include the possible signal "contamination" with Mg^{2+}-induced fluorescence, the need for taking measures to avoid the appearance of movement artifacts (see "Enzymatic dissociation and hand dissection") in the Ca^{2+} transients and a more complex calibration procedure.

Ratiometric dyes, on the other hand, may show both intensity and spectrum changes when bound to Ca^{2+}. The main examples of this group are: Fura-2 (Baylor and Hollingworth 1988; Westerblad and Allen 1991), Indo-1 (Chin and Allen 1998) and Mag-Fura-5 (Delbono and Stefani 1993; Szentesi et al. 1997). Their main disadvantages include the need for ultraviolet excitation and a more complex instrumentation. Also, with the exception of Mag-Fura-5, they may saturate and seem not to reliably track Ca^{2+} kinetics in skeletal muscle. One of the most important advantages of this group of dyes is the possibility of recording Ca^{2+} transients free of movement artifacts (Kao et al. 2010; Bruton et al. 2012) and their suitability for measuring myoplasmic basal Ca^{2+}. Other advantages include their minimum Mg^{2+} affinity and the possibility of calibration disregarding the dye concentration.

Although for tracking Ca^{2+} changes focused on particular subcellular structures, some Ca^{2+} dyes may be used (Rhod-2, Mag-Fluo-4; Fluo-5N, CalciumOrange-5N; Bruton et al. 2003; Brochet et al. 2005; Bolaños et al. 2008; Kao et al. 2010), the genetically-encoded Ca^{2+} sensors seem to be now a better choice (Palmer and Tsien 2006; Rudolf et al. 2004; Tang et al. 2011; Wang et al. 2012).

As illustrated in Fig. 1, the kinetics of the single Ca^{2+} transients largely depend on the kinetic properties of the dye

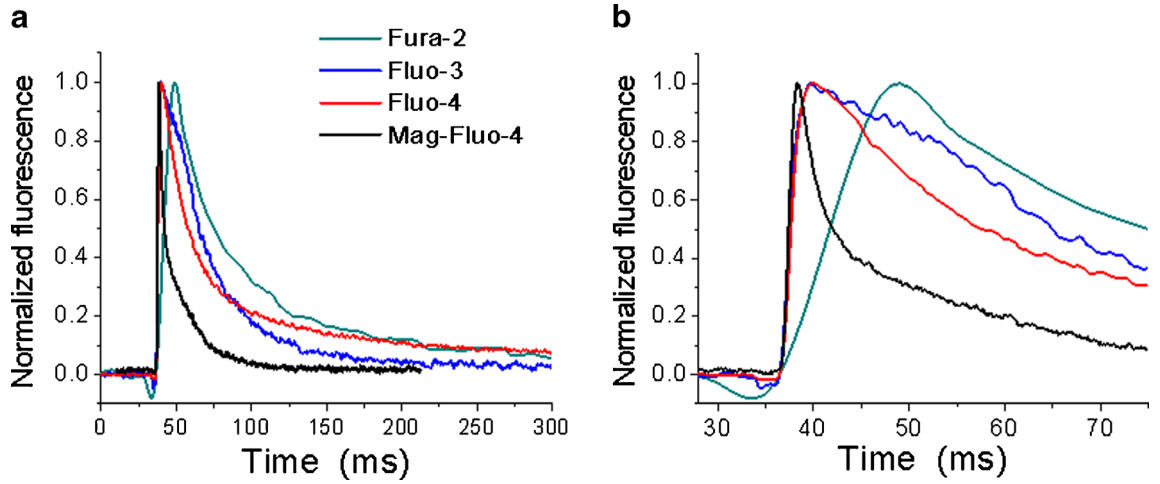

Fig. 1 Comparison of single Ca^{2+} transients' kinetics recorded in muscle fibres obtained by enzymatic dissociation of flexor digitorum brevis muscles from adult mice. Different cells were loaded with each of the Ca^{2+} dyes indicated in the figure and electrically stimulated. Ca^{2+} transients were recorded in an inverted fluorescence microscope using the appropriate set of filters, a photomultiplier and a Nikon amplifier. In (**a**), clear kinetic differences can be recognized, mostly derived from the different dissociation constants of the dyes used, being the fastest signal that obtained with Mag-Fluo-4 (*black trace*) and the slowest one that obtained with Fura-2 (*green trace*). In (**b**), the records are shown in an expanded time scale to better illustrate differences in the rising part of the signal

used. In this figure, Mag-Fluo-4 is the only low-affinity Ca^{2+} dye and shows the best kinetics for tracking skeletal muscle Ca^{2+} transients. In any case, the researcher should know how to deal with the limitations of a given dye and also how to exploit its advantages, since the ideal Ca^{2+} indicator is still missing.

Tension measurements

For tension measurements, small bundles of fibres and manually isolated fibres are used (Caputo and Giménez 1967; Lännergren and Westerblad 1987; Baylor and Hollingworth 2003; Bruton et al. 2003; Edman 2005). Therefore, muscle fibres remain attached to their tendons. Isolated fibres or bundles are horizontally mounted on the experimental chamber and attached with small aluminum clips, on one end to a tension transducer and on the other to a hook attached to the chamber. Contractions are produced by suprathreshold stimulation through electrodes. Simultaneous measurements of Ca^{2+} and tension can be obtained (Westerblad and Allen 1991; Bruton et al. 2003; Baylor and Hollingworth 2003; Calderón et al. 2011). This allows to calculate, for instance, myofibrils' sensitivity to Ca^{2+} in intact fibres, or to follow the changes of both variables during muscle fatigue. Recently, a biological adhesive was successfully used to attach dissociated fibres to a tension transducer (Ward et al. 2011) ,opening up a large number of possibilities with this cellular preparation.

Electrophysiology

Membrane ionic current measurements, intramembrane charge movement recordings and studies of voltage dependence of Ca^{2+} release, through membrane voltage-clamp techniques are of great importance for the study of numerous aspects of physiology and physiopathology of skeletal muscle (Delbono and Stefani 1993; Szentesi et al. 1997; Hernández-Ochoa and Schneider 2012). Currently, the triple vaseline gap voltage clamp, the whole-cell patch-clamp and the silicone-clamp techniques are the most used for recording Ca^{2+} transients under controlled membrane potential conditions, in myotubes, intact or cut isolated fibres. In adult fibres, because of their length, it is convenient to use the vaseline-gap and silicone-clamp techniques to isolate areas of the fibres and thus restrict current measurements to small portions of the fibres. In the obtained records, the amplitude and voltage dependence are the most important variables to analyze. Depolarization occurs from a set voltage (holding potential) usually from –80 to –100 mV, to a variable voltage. Depolarization records from –80 to –10, 0, +10 and +30 mV are usually obtained (Delbono and Stefani 1993; Szentesi et al. 1997; Beam and Franzini-Armstrong 1997; Pouvreau et al. 2007a; Hernández-Ochoa and Schneider 2012).

In a different technique, by inserting purified proteins into artificial lipid bilayers and activating the passage of ions through them, it is possible to study their channel properties and to estimate their conductance, open probability, selectivity and drugs effects (Smith et al. 1988; Goonasekera et al. 2007).

Confocal laser scanning microscopy and super-resolution microscopy

Confocal laser scanning microscopy (CLSM), and, more recently, multiphoton microscopy, have allowed us to perform temporal and spatial precise analysis of ECC-related physiological phenomena at a subcellular level, besides the suitability

of CLSM for structural studies (Cheng et al. 1993; Escobar et al. 1994; Rudolf et al. 2004; Brochet et al. 2005; Bolaños et al. 2008; Casas et al. 2010; Figueroa et al. 2012). Cells may be loaded with indicators such as di-8-ANEPPS and FM 1-43 to label sarcolemma and T-tubules and record transmembrane voltage; Rhod-2, Fluo-3 and Fluo-4 for cytoplasmic Ca^{2+}; Endoplasmic Reticulum-Tracker Green (ERTG) for SR and MitoTracker Green, Rhod-2, CalciumOrange-5N, JC-1 and Tetramethyl rhodamine ethyl ester (TMRE) for different mitochondrial studies (Farkas et al. 1989; Escobar et al. 1994; Rudolf et al. 2004; Bolaños et al. 2008; Calderón et al. 2009; Casas et al. 2010; Manno et al. 2013). Fluorescent molecules-coupled antibodies and fluorescent proteins-coupled protein constructs are also visualized using CLSM. Confocal microscopy has the following advantages over optical microscopy and wide-field fluorescence: (1) higher z-resolution and better contrast, it allows us to obtain better structural (i.e. T tubules, SR or mitochondria) and functional information (Ca^{2+} and transmembrane potential imaging), (2) allows us to obtain images for further analysis (temporary changes, fluorescence intensity measurements, and morphometric measurements, among others), (3) allows us to simultaneously register different structures or events for further joint analysis, and (4) allows us to perform optical sections, which is especially important to determine the location of fluorescent compounds, and to perform three-dimensional reconstructions, in addition to producing multidimensional images according to time (*xt* or *xyt* modes). One limitation of CLSM has been its low temporal resolution when studying kinetics of Ca^{2+} transients with fast Ca^{2+} dyes. However, recently, some authors overcome this limitation, and were able to track Ca^{2+} transient's propagation with fast scanning confocal microscopy in rat fibres (Edwards et al. 2012).

Additionally, the use of CLSM combined with new techniques such as FRET (Förster resonance energy transfer) and SEER (Shifted excitation and emission ratioing) have allowed, among other findings, to study conformational coupling between DHPR and RyR and to obtain images which allow to quantify the Ca^{2+} inside the SR or dynamically image transmembrane voltage (Papadopoulus et al. 2004; Launikonis et al. 2005; Manno et al. 2013).

Although the theoretical background for super-resolution microscopy has been developed during the last two decades (Hell and Wichmann 1994; Klar et al. 2000; Gustafsson 2000; Schermelleh et al. 2010), this technique became commercially available very recently and only a few works have used it to study skeletal muscle ECC (Rausch et al. 2013), besides cardiac ECC (Jayasinghe et al. 2012; Scriven et al. 2013; Wong et al. 2013). The main advantage of the technique is providing a *xy*-resolution between 30 and 120 nm, which recently allowed some authors to recognize a new pattern of RyR clustering in mouse FDB fibres (Rausch et al. 2013). The main disadvantages include the need for non-standard dyes in some devices, still limited temporal resolution and high costs.

Other procedures

(1) Electron microscopy (standard, metal-shadowed, cryomicroscopy and three-dimensional reconstructions) has generated important information on the triad structure, membrane systems (T-tubules and SR), DHPR-RyR interaction and SR-mitochondria relationship (Block et al. 1988; Serysheva et al. 2007; Boncompagni et al. 2009; also see below). Because there are no X-ray diffraction patterns of the whole DHPR and RyR proteins, due to the difficulties of crystallizing them, cryomicroscopy of isolated particles and three-dimensional reconstructions have been used for structural and functional characterization. However, the resolution obtained does not allow a clear non-ambiguous secondary structural assignment for some proteins (see below).

(2) Several molecular biology and genetic engineering techniques have provided valuable information on skeletal muscle in normal and pathological conditions, since they have allowed the functional evaluation of the expression (or absence) of proteins involved in ECC, i.e: (1) knock-out mice for some proteins, including dysgenic mice lacking the subunit α-1 of DHPR, and dyspedic mice, lacking RyR1 (Buck et al. 1997; Beam and Franzini-Armstrong 1997; Prosser et al. 2008). Mice lacking CSQ or other minor ECC proteins have been under research during the past years (Paolini et al. 2007; Royer et al. 2010; Mosca et al. 2013); (2) gene silencing, as in the codifying gene for SR integral protein, JP-45, proving its importance for functional expression of DHPR (Anderson et al. 2006); and (3) production and extraction of recombinant proteins with high qualitative and quantitative performance in adult mammalian skeletal fibres, which has allowed researchers to evaluate the effect of mutated ion channel's expression and endogenous protein over-expression, such as DHPR, on ECC (DiFranco et al. 2006, 2011).

Particular issues on ECC

Dihydropyridine receptor-ryanodine receptor structure and coupling

There is evidence indicating a mechanical, bidirectional conformational coupling between DHPR and RyR in the skeletal muscle (Nakai et al. 1996; Ávila and Dirksen 2000; Fill and Copello 2002; Paolini et al. 2004), which would be mediated by regions of the internal loop joining DHPR's II and III transmembrane domains and a specific short RyR region (Tanabe et al. 1990; el-Hayek et al. 1995; Leong and MacLennan 1998; Casarotto et al. 2006), although several studies suggest that there are multiple RyR1 regions interacting with DHPR

(Protasi et al. 2002) and multiple DHPR subunits doing so with the RyR1 (Papadopoulus et al. 2004).

Two important and related developments opened the way for clarifying, at a molecular level, the roles of DHPR and RyR, and their relationship in ECC: (1) the cloning and sequencing of the complementary DNA (cDNA) that encoded for the $\alpha 1$ subunit of DHPR (Tanabe et al. 1987), and of the skeletal muscle RyR (Takeshima et al. 1989) and (2) the availability of mice with genetic alterations at level of the DHPR, or at level of the RyR. In the case of DHPR, it was shown that mice with a mutation in the $\alpha 1$ subunit of this molecule, dysgenic mice, lacked the L-type Ca^{2+} current and presented greatly reduced intramembrane charge movement (Beam et al. 1986). These results provided the first hint that DHPR could have a dual function, serving both as voltage-activated Ca^{2+} channels and as voltage sensors for ECC, an idea reinforced by the simultaneous evidence provided by Ríos and Brum (1987) that the DHPR were the molecules generating the intramembrane charge movement, which represents the electrical manifestation of the voltage sensors operation. Definitive evidence in favour of this idea was obtained by the fact that expressing skeletal muscle DHPR $\alpha 1$ subunit in dysgenic myotubes restored both the slow Ca^{2+} current (Tanabe et al. 1988) and the intramembrane charge movement (Adams et al. 1990).

An elegant experimental strategy was developed to determine the regions of the DHPR important for ECC, based on the differential sensibility of skeletal and cardiac ECC to extracellular Ca^{2+} and the use of myotubes of dysgenic animals that did not express DHPR (Tanabe et al. 1990). Injection of cDNA codifying for the cardiac isoform of the DHPR reproduced the cardiac type of ECC, that required the presence of external Ca^{2+} for contracting in response to electrical stimulation and was disrupted by Cd^{2+} poisoning. Injection of cDNA, codifying for skeletal type DHPR, reproduced the skeletal ECC with injected myotubes contracting in the absence of external Ca^{2+} and not sensitive to Cd^{2+}. Further experiments using expression plasmids, in which only the loops between I-II and II-III domains of the DHPR were of skeletal origin, showed that the skeletal region between repeats II and III was a sufficient determinant for skeletal type ECC.

Most DHPR–RyR interaction studies have been based on site-directed mutagenesis of amino acids located in specific domains. However, the movements of different domains involved in such interactions are not completely clear, due to lack of information from crystallography or magnetic resonance studies of both channels under different circumstances. Although the three-dimensional structure of the isolated DHPR II-III loop is already known (Casarotto et al. 2006), it is still uncertain if it assumes a different structure when binding with RyR or if it remains unchanged in relation to the complete DHPR structure. The β_{1a} subunit of the DHPR has also been shown to be important for ECC coupling, and ongoing work is devoted to clarify its role in interacting with and modulating RyR, as well as its role in the adecuate targeting (i.e. forming tetrads) of DHPR to the T-tubules (Pérez et al. 2013; Rebbeck et al. 2013).

So far, most structural information on DHPR and RyR structures comes basically from cryomicroscopy studies and three-dimensional reconstructions (Wagenknecht et al. 1989; Ludtke et al. 2005; Samsó et al. 2005, 2009; Serysheva et al. 2007). Despite the fact that several teams are solving the structure of both proteins, only recently have sub-nanometric resolution images been published (9.6 Å for the whole RyR (Ludtke et al. 2005) and 2.5 Å for the structure generated by the 559 aminoacids of the N-terminal region of the RyR (Tung et al. 2010). The authors have specially focused on the RyR's pore structure at its closed state. Ludtke et al. (2005) identified 5 α helices per monomer in the transmembrane region and suggested that helices 1 of each subunit form the pore, and helices 2, the selectivity filter. Samsó et al. (2005) suggested the existence of at least 6 transmembrane helices per monomer, and described in some detail the structures known as columns (inner branches), which connect the transmembrane portion with the great cytosolic domain. It is possible that the pore's structure is similar to some already published K^+ channel structures (Doyle et al. 1998; Jiang et al. 2002; Ludtke et al. 2005; Samsó et al. 2005, 2009). Recently, an image of the open channel (at 10.2 Å) was obtained and moderate conformational changes were recognized when compared to the closed state. The cytoplasmic domains move outward and the three constrictions identified by the authors in the ion pathway show a wider central passage (Samsó et al. 2009).

The highest resolution obtained (9,6 Å in RyR1 and 30 Å in DHPR) is not clear enough to show details such as: (1) the exact molecular mechanism involved in receptor's interactions, (2) presence, distribution and movement of α helices and β sheets to explain, for instance, how the signal is transmitted from the DHRP to the RyR and how the information from the RyR regulators is transmitted from somewhere in the protein to the pore, to allow SR Ca^{2+} release, (3) delimitation of the different subunits in each protein, and (4) the number of RyR helices going through the SR membrane, since the proposed number of transmembrane helices varies between 4 and 10–12 per monomer (Takeshima et al. 1989; Zorzato et al. 1990; Samsó et al. 2009).

The 2.5-Å resolved structure of the N-terminal domains of the RyR shows that it is located in the cytoplasmic portion of the protein, and the domains orientation likely represents the closed state of the channel (Tung et al. 2010). In this region, the positions of multiple disease-mutations and some regions of intersubunit interactions can be mapped, but it is likely that, in spite of the high resolution, no clear information on the above questions will be obtained by this structure. The answer to these and other issues relies on obtaining a complete structural image of both DHPR and RyR channels with at least a 3-6-Å resolution.

Isolated protein images do not necessarily reflect the structure of complexes such as the triad. Images of isolated rabbit triads (Wagenknecht et al. 2002) with an approximate resolution of 6 nm, obtained through several techniques, confirmed findings previously made by other authors (Franzini-Armstrong and Porter 1964; Block et al. 1988; Franzini-Armstrong and Jorgensen 1994; Franzini-Armstrong 1999) and drew attention to the existence of some new structural characteristics: (1) there could possibly be a 5-nm-wide structure under the SR's internal membrane, whose nature is still unknown, (2) CSQ particles seem to be interconnected within the SR, (3) inside the T-tubule's lumen, there appear to be structures whose molecular nature is still unknown but could play a structural role, and (4) the presence of structures covering the distance from the RyR's cytoplasmic region all the way to the T-tubule was found, which cannot be categorized in a clear protein domain, but could be DHPR loops. In spite of the advantages of this technique, the results obtained could be affected by artifacts during the sample processing, and by the low resolution of the images.

Aside from the above, we still need to know the three-dimensional structure of triadic major and minor proteins, including previously identified and some still unidentified ones. Although some models of the triadic complex have been recently put forward and the specific domains and aminoacids involved in intra- and interprotein interactions are being identified (Wagenknecht et al. 2002; Treves et al. 2009; Fourest-Lieuvin et al. 2012; Boncompagni et al. 2013), the huge amount of proteins and expected interactions among them that regulate ECC, and which may also function as molecular determinants of the shape of the membranous components of the triad, makes the work on these issues still far from finished. Nowadays, despite almost three decades of research on the DHPR-RyR coupling issue, two basic questions remain unanswered: how are different signals transmitted from the bulk of the channel to the pore, and how is the ion gating mechanism of RyR?

ECC and fibre types

In 1873, Ranvier stated that there were pale and red muscles, being different mainly regarding their contraction speed (Ranvier 1873). Decades later, a more complete profile of both types of fibres was generated and new fibre types were described (Dubowitz and Pearse 1960; Brooke and Kaiser 1970; Bär and Pette 1988; Schiaffino et al. 1989). Since four fibre types based on myosin heavy chain (MHC) presence have been described, I, IIA, IIX/D and IIB (there are also hybrid fibres), one important question that raises is whether ECC proteins and Ca^{2+} transient kinetics differ among all fibre types.

The molecular machinery involved in ECC is qualitatively (different isoforms) and quantitatively (different amount of proteins or different kinetics) different between slow and fast-twitch fibres (Table 1). The information presented in this table discriminates between slow- and fast-twitch fibre types, since the information is not available for each MHC-based fibre type. A great variability regarding the proteins involved in both Ca^{2+} release and Ca^{2+} removal from the myoplasm is evident. The most striking difference is found in parvalbumin concentration, ranging from almost zero in the slowest fibres up to 1 mM in the fastest fibres.

The differences mentioned in Table 1 constitute the biochemical and structural bases for the Ca^{2+} transients variability found among fibre types (Eusebi et al. 1980; Carroll et al. 1997; Bottinelli and Reggiani 2000; Baylor and Hollingworth 2003; Reggiani and te Kronnie 2006; Calderón et al. 2009, 2010).

Single Ca^{2+} transients obtained using Mag-Fluo-4 can be classified according to their kinetics into two different morphologies (Calderón et al. 2009, 2010). One morphology, found in fibres type I and IIA, is slower, wider and of less amplitude and was called morphology type I (MT-I). The other morphology, found in fibre types IIX/D and IIB, is faster, narrower and of higher amplitude and was called morphology type II (MT-II). An example of the Ca^{2+} transients found in different fibre types from mice muscles is illustrated in Fig. 2. Since only minor differences can be recognized between fibres type I and IIA, for the sake of clarity, we have pooled in Table 2 the kinetics of a large number of MT-I fibres obtained from soleus muscles. The data are compared with the pooled values obtained in EDL fibres, since all of them are MT-II. Significant differences can be recognized in all parameters describing amplitude, rising and decay kinetics between soleus and EDL signals.

Tetanic Ca^{2+} transients of the fibres type I and IIA show a staircase morphology (MT-I). In these transients, the decay can be fitted by a single exponential function. On the other hand, fibres type IIX/D and IIB have tetanic Ca^{2+} transients whose decay is better fitted with a biexponential function (MT-II) (Calderón et al. 2009, 2010).

In general, regarding Ca^{2+} transients and fibre types, it can be concluded that transient's kinetics show a continuum from the slowest kinetics, obtained in the type I fibres, to the fastest ones, obtained in types IIX and IIB. Remarkably, type IIA fibres are fast regarding Ca^{2+} release and slow regarding Ca^{2+} clearance (Calderón et al. 2009, 2010). The differences in kinetics of the Ca^{2+} twitch and tetanic transients seem to underlie the different kinetics of contractile characteristics of slow and fast muscles (Calderón et al. 2010).

One application of the easily recognizable difference found between MT-I and MT-II records is the possibility of functionally recognizing fibre types while still alive during physiological experiments in which Ca^{2+} transients are recorded (Calderón et al. 2011, unpublished results).

Ongoing work has addressed the mechanisms responsible for the Ca^{2+} transient's kinetics differences. The biochemical differences shown in Table 1 and new results suggest that

Table 1 Biochemical and structural differences in ECC between slow and fast-twitch mammalian fibre types

Proteins	Slow-twitch fibres		Fast-twitch fibres		References
	Isoform	Content/ kinetics	Isoform	Content/ kinetics	
DHPR	α-1S	+	α-1S	+++	Hollingworth and Marshall 1981; Lamb and Walsh 1987; Franzini-Armstrong et al. 1988.
RyR	RyR1	+	RyR1	+++	Franzini-Armstrong et al. 1988; Appelt et al. 1989; Damiani and Margreth 1994.
PV	α-PV	– to +	α-PV	++ to ++++	Heizmann et al. 1982; Leberer and Pette 1986; Schmitt and Pette 1991; Füchtbauer et al. 1991.
SERCA	SERCA2a	+	SERCA1a	+ to ++	Leberer and Pette 1986; Dulhunty et al. 1987; Ferguson and Franzini-Armstrong 1988; Periamasy and Kalyanasundaram 2007.
Phospholamban	Phospholamban	+	Phospholamban	–	Jorgensen and Jones 1986.
Sarcolipin	Sarcolipin	+	Sarcolipin	+++	Odermatt et al. 1998.
Calsequestrin	CSQ*$_{fast}$ and CSQ*$_{cardiac}$	+	CSQ*$_{fast}$	+	Leberer and Pette 1986; Damiani and Margreth 1994.
SRP-27	SRP-27	+	SRP-27	+++	Bleunven et al. 2008
NCX	NCX1	++	NCX1 and 3	+	Fraysse et al. 2001; Hudecova et al. 2004
TnC, TnI, TnT	TnC slow TnI slow TnT slow	+	TnC fast TnI fast TnT fast	++	Bottinelli and Reggiani 2000.
mATPase	Type I	+	Types IIA, IIX/D and IIB	++ to +++	Dubowitz and Pearse 1960; Brooke and Kaiser 1970; Bär and Pette 1988; Schiaffino et al. 1989; Bottinelli and Reggiani 2000.

DHPR dihydropyridine receptors; *RyR* ryanodine receptors; *PV* parvalbumin; *SERCA* sarcoendoplasmic reticulum Ca^{2+} ATPase; *CSQ* calsequestrin; *SRP-27* sarcoplasmic reticulum protein-27 kDa; *NCX* Na^{+}/Ca^{2+} exchanger; *TnC* troponin C; *TnI* troponin I; *TnT* troponin T; *mATPase* myofibrillar A-band adenosine triphosphatase

– indicates absence of the protein; ++ indicates approximately twofold; +++ between twofold and tenfold; ++++ more than tenfold, in all cases compared to a reference of +

higher DHPR and RyR content explain differences in the rising phase of the Ca^{2+} transients and that PV content and SERCA kinetics explain most of the decay differences (Calderón et al. 2009, 2010, unpublished results). Other mechanisms such as mitochondria and NCX only play a minor role in explaining the differences (Calderón et al., unpublished results).

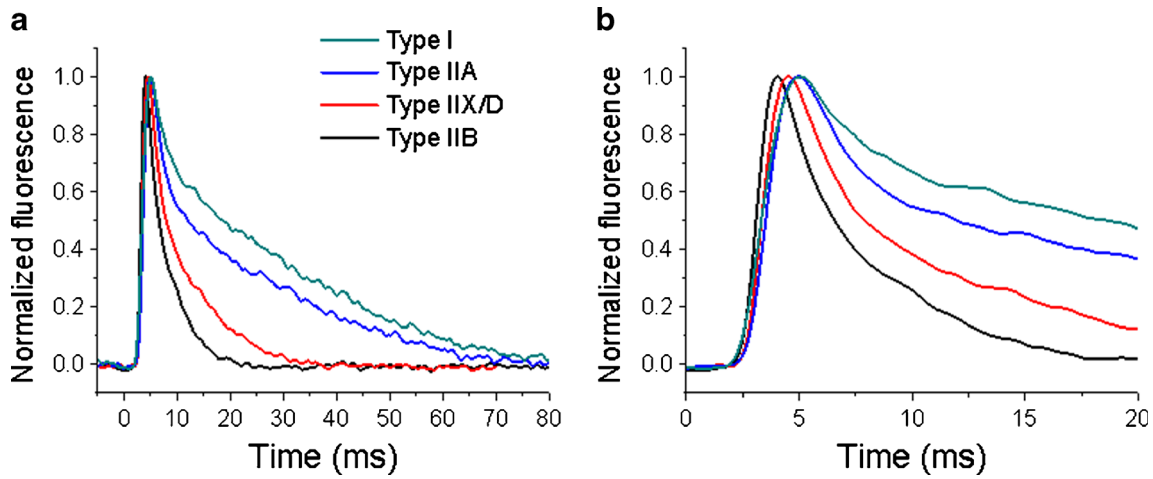

Fig. 2 Time course of single Ca^{2+} transients of different fibre types obtained by enzymatic dissociation of extensor digitorum longus and soleus muscles from adult mice and typed by polyacrylamide gel electrophoresis. The cells were loaded with Mag-Fluo-4. A pattern can be recognized, with the pair I and IIA being the slowest and the pair IIX/D and IIB being the fastest both during decay (**a**) and rise (**b**)

Table 2 Kinetic parameters of soleus and EDL single Ca^{2+} transients from adult mice

Muscle	*n*	ΔF/F	10-90 % Rise time (ms)	Half-width (ms)	Decay time (ms)	Time constants (ms)		A_1 (%)	A_2 (%)	ΔF/RT
						t_1	t_2			
Soleus*	45	0.51±0.03	1.56±0.04	15.73±0.95	61.35±2.41	3.14±0.11	43.63±2.23	27.17±1.32	72.83±1.32	0.34±002
EDL	26	0.65±0.02	1.08±0.03	4.07±0.2	15.92±0.88	1.58±0.07	9.99±0.67	39.45±1.47	60.55±1.47	0.61±0.03

Values are mean ± SEM

EDL Extensor digitorum longus; *A1* amplitude of fast component of decay; *A2* amplitude of slow component of decay, *ΔF/RT* ratio of amplitude to rise time

*$p<0.05$ for all comparisons between each one of the parameters (i.e. ΔF/F of soleus vs. EDL, 10–90 % rise time of soleus vs. EDL, etc.). From: Calderón 2013, with permission

ECC and muscle fatigue

The transient and progressive decrease in skeletal muscle performance during continuous stimulation is known as fatigue (Fitts 1994; Allen et al. 2008). This is a complex phenomenon with central and peripheral components (Bigland-Ritchie and Woods 1984; Abbiss and Laursen 2005; Allen et al. 2008). Central fatigue involves events occurring prior to the ECC phenomenon, while peripheral fatigue involves events including ECC and other phenomena occurring inside the muscle fibre as a consequence of its activation.

Although different models to study muscle fatigue show different complexity, it seems that data gathered with a relatively simple model such as the one of isolated fibres can be used to understand some fatigue mechanisms in more complex models (Place et al. 2010). The most important addressed questions on fatigue are: (1) what is the cause of skeletal muscle fatigue, and (2) what is the mechanism by which muscle fatigue develops?

In several preparations, fatigue has been shown to be mainly peripheral (Grabowski et al. 1972; Bigland-Ritchie and Woods 1984; Moussavi et al. 1989; Kent-Braun 1999; Place et al. 2008, 2010), explained in part by alterations in the Ca^{2+} release mechanism (Grabowski et al. 1972; Allen et al. 1989; Westerblad and Allen 1991). In the same way, alterations of the SERCA function and slowing of single-twitch and tetanic relaxation have been demonstrated as a result of fatigue (Gollnick et al. 1991; Westerblad and Lännergren 1991; Westerblad and Allen 1993; Green 1998; Leppik et al. 2004), but the slowing of relaxation cannot be totally explained by the decreased SERCA pumping rate (Westerblad andAllen 1993). Other phenomena, such as a decrease in the intra-SR Ca^{2+} content and the damage of membrane systems involved in ECC, may also be implicated (Takehura et al. 2001; Tupling 2004). An alteration of the inactivation of the Ca^{2+} release mechanism seems not to mediate Ca^{2+} release disturbances found in fatigue (Calderón et al. 2011).

Although a wealth of evidence supports the concepts according to which both a decrease in AP amplitude or ATP depletion can be ruled out as causes of fatigue (Luttgau 1965; Grabowski et al. 1972; Nassar-Gentina et al. 1978; Moussavi et al. 1989; Allen et al. 2002), recent findings, suggesting that localized ATP decrease close to critical cellular regions may be important for fatigue (Allen et al. 1997) or that an AP decrease in fatigued fibres can be measured by SEER (Manno et al. 2013), warrant further research on these topics.

ECC alterations during fatigue are strongly supported by the fact that, in a fatigued muscle, tension development can be reactivated by caffeine (Grabowski et al. 1972; Allen et al. 1989) and because it was actually proved that fatigued fibres have reduced tetanic Ca^{2+} transients (Westerblad and Allen 1991; Calderón et al. 2011). The ECC alteration, however, is more notorious in fast-twitch fibres compared to slow ones (Calderón et al. 2011), as expected by the known profile of fatigability of the different fibre types (Burke et al. 1973; Petrofsky and Lind 1979; Westerblad and Allen 1991; Bruton et al. 2003).

Metabolic factors, such as changes in Ca^{2+} itself, ATP, phosphocreatine (PCr), H^+, Mg^{2+}, Pi and reactive oxygen species (ROS) concentrations observed during fatigue may be involved in altering the ability of the SR to release and reuptake Ca^{2+} (Lamb 2002; Tupling 2004; Allen et al. 2008; Calderón-Vélez and Figueroa-Gordon 2009).

Lactate accumulation and acidosis were the most claimed factors in explaining the decrease in muscle performance; however, its role has been challenged, although not without some controversy (Hill and Kupalov 1929; Westerblad 1999; Westerblad et al. 2002; Pedersen et al. 2004; Bangsbo and Juel 2006), even when pH values as low as 6.5 and lactate increase have been documented in several works as related to fatigue (McCully et al. 1991; Lindinger and Heigenhauser 1991; Kent-Braun et al. 1993; Kent-Braun 1999). pH reduction seems to decrease fibre's maximum strength, myofibrillar Ca^{2+} sensitivity and SR ability to release Ca^{2+} (Rousseau and Pinkos 1990; Fitts 1994; Lamb 2002; Knuth et al. 2006).

Another possible mechanism involving ECC as a potential fatigue site could be that the increase in basal myoplasmic Ca^{2+} ($Ca^{2+}{}_{myo}$) found during the development of fatigue (Westerblad and Allen 1991; Caputo et al. 1994) may disrupt DHPR–RyR interaction (Lamb 2002). This seems to be associated to an

alteration of the fibre to produce tension although the SR's ability to deal with Ca^{2+} does not become altered (Lamb et al. 1995; Verburg et al. 2005). SERCA function alteration (Green 1998; Westerblad and Lännergren 1991; Westerblad and Allen 1993; Leppik et al. 2004; Tupling 2004) could explain the persisting increase of Ca^{2+}_{myo} leading to the ECC disruption and subsequently to a decrease in Ca^{2+} release after each stimulus. Opposite to this, it was suggested that a more important factor for the production of uncoupling is the localized increase of Ca^{2+} near the triad, associated to each tetanus, instead of the increase of basal Ca^{2+}_{myo} (Verburg et al. 2006).

Although free myoplasmic Mg^{2+} increases during repeated stimulation (Westerblad and Allen 1992) and it is an inhibitor of the SR Ca^{2+} release (Meissner 1984), Mg^{2+} does not have an important fatiguing effect unless reaching very high concentrations, which do not occur in a fatigued muscle fibre (Westerblad and Allen 1992; Lamb and Stephenson 1994).

Even though theoretical considerations could also support a role for ROS and free radicals in fatigue (Barclay and Hansel 1991; Sen 1995; Reid 2001; Darnley et al. 2001), by altering Ca^{2+} release from the SR (Brotto and Nosek 1996; Oba et al. 2002; Hidalgo 2005; Bruton et al. 2008), or by reducing myofibril's sensitivity to Ca^{2+} in mammalians (Moopanar and Allen 2005, 2006; Bruton et al. 2008; Reardon and Allen 2009), conflicting results are available on whether these species are actually produced during a repetitive stimulation protocol in different experimental preparations (Davies et al. 1982; Reid et al. 1992; Kanter et al. 1993; Bruton et al. 2008; van der Poel et al. 2008) and thus on whether their effect would be physiologically relevant to explain fatigue-induced ECC alterations in mammalians. The issue of the relationship among high temperature-ROS and fatigue seems to be illustrative. High temperature may increase ROS production in rodents, and ROS may induce fatigue (van der Poel et al. 2008; Reardon and Allen 2009; Michaelson et al. 2010; see also Place et al. 2009); however, high temperature did not alter fatigability in some reports (Place et al. 2009; Reardon and Allen 2009).

Nowadays, we are not certain about what are the exact physiological targets of free radicals and ROS in fatigue, what is the main source of ROS during repetitive contraction (see van der Poel et al. 2008; Michaelson et al. 2010), or if the lipid peroxidation may explain the alterations of the different ECC-related proteins affected in the fatigue state and which are the chemical species involved. Until now, the results depend on the animal species, the techniques used for detecting ROS and the experimental protocols applied (muscle preparation, stimulation duration and temperature). Some recently developed tools may help solve technical problems with the detection of intracellular ROS and their role in muscle fatigue (Pal et al. 2013).

During the last two decades, an increase in free myoplasmic Pi has emerged as an important cause of fatigue. Pi meets important requirements to be considered as cause of ECC fatigue. Firstly, its change has a time course similar to the time course of the fatigue-induced Ca^{2+} kinetic changes (Westerblad and Allen 1991; Calderón et al. 2011). Changes in Pi can start early during the fatigue development, although are more notorious during the second half of the stimulation periods (Bergström and Hultman 1988; Moussavi et al. 1989; Kent-Braun et al. 1993; Kent-Braun 1999); several studies have shown that Pi is important for the early changes in the kinetics of Ca^{2+} signals (Dahlstedt et al. 2001; Westerblad et al. 2002). Secondly, an increase in Pi can explain the most important fatigue-induced Ca^{2+} kinetic changes (Westerblad and Allen 1991; Calderón et al. 2011). Pi can regulate both the release of Ca^{2+} and the function of SERCA (Duke and Steele 2000; Dahlstedt et al. 2001; Westerblad et al. 2002) and it seems to favor Ca^{2+} deposit inside the SR, in the form of Ca^{2+} phosphate, which diminishes the amount of Ca^{2+} available for release (Fryer et al. 1995; Dutka et al. 2005). It can also decrease myofibrillar Ca^{2+} sensitivity (Fitts 1994; Westerblad et al. 2002). Thirdly, the differential change in Pi observed in slow and fast fibres during contraction gives support to the differential alteration seen in the rising and decay phase of tetanic Ca^{2+} transients demonstrated in different fibre types during fatigue (He et al. 2000; see Calderón et al. 2011). And, fourthly, Pi concentration manipulation modifies in the same way the fatiguing properties of different muscles (Dahlstedt et al. 2001; Westerblad et al. 2002).

The development and availability of Pi fluorescent indicators would be of great impact to prove a direct relationship between intracellular changes of the metabolite and the phenomena known to occur during fatigue. Also, to confirm the role of Pi on muscle fatigue, the management of and the sensitivity to Pi alterations by the different fibre types in different experimental models should be evaluated.

Other questions are still open, such as: (1) what are the kinetics and mechanisms of muscle fatigue in developing or ageing fibres, (2) which mechanisms explain the fatigue-resistant properties found in slow *vs.* fast-twitch fibres, and (3) what is the role of ECC regulators or minor proteins in muscle fatigue (see Prosser et al. 2010)?

Contractile potentiators

The early observation by Kahn and Sandow (1950) that nitrate augmented the twitch response of frog skeletal muscle led to the demonstration by Hodgkin and Horowicz (1960) that nitrate and other anions, of the lyotropic series $SCN > I > NO_3 > Br > Cl$, shifted, in this order of potency, the relationship between contractile force and membrane potential, toward more negative potentials, thus reducing the contractile threshold, that in frog fibres is around –50 mV (Sandow 1964; Kao and Stanfield 1968). The lyotropic series describes the degree of adsorbability of these anions on the membrane external surface that cause a change in the density of fixed charges (Hodgkin and Horowicz 1960; McLaughlin et al.

1975), altering the surface potential and the potential gradient through the fibre membrane. Of particular importance is the chaotropic anion perchlorate, ClO_4^-, that causes the largest change in the contractile threshold, potentiating manifold twitch tension (Foulks et al. 1973; Gomolla et al. 1983). A group of divalent heavy metals, such as Zn^{2+}, Be^{2+}, Pt^{4+} and others including uranil ions $(UO_2)^{2+}$, also act as twitch potentiators, by a selective block of the K^+ conductance, that prolongs the duration of the AP, overcoming the non-specific effects due to their adsorbability on the surface membrane (Sandow and Isaacson 1966). Sandow et al. (1965) proposed that both types of compounds enhanced the twitch, by prolonging a parameter, called the mechanically effective period (MEP), defined as the period during which the AP causes the release of an amount of Ca^{2+}, greatly exceeding that necessary for a twitch response (Sandow 1964). MEP is increased either by lowering the contractile threshold (type A potentiators, like the lyotropic anions), or by prolonging the AP (type B potentiators, like the divalent heavy metals Zn^{2+}, Cd^{2+}, etc.). Other compounds, like caffeine, quinine and quinidine, that also potentiated contraction, were initially classified as type C, since they seemed to act by a combination of both type A and type B mechanisms (Sandow 1964), but later, however, they were classified as type A, due to their sizeable effect on the contractile threshold. In all cases, prolongation of the active state could explain twitch potentiation, according to the classical view as type A potentiators (Hill 1949; Sandow 1965). While the prolongation of the AP remains the undiscussed mechanism of action for type B potentiators, the mechanism of action of type A potentiators requires some revision, in view of the increased knowledge about ECC. Nowadays, it is generally accepted that, in skeletal muscle, Ca^{2+} release from the SR is triggered by the mechanisms described in "The excitation–contraction coupling (ECC) mechanism in skeletal muscle".

The demonstration that lyotropic anions had similar effects on intramembrane charge movement and ICa^{2+} supported the idea that the DHPR serve as voltage sensor for ECC (Ríos and Brum 1987; Delay et al. 1990). In contrast, ClO_4 at concentrations lower than 10 mM selectively affected charge movement and contractile activation, without affecting activation of the AP (Ina) and activation of the delayed rectifier (Ik). SCN, the most potent of the lyotropic anion series, was found to be less selective than ClO_4^-. In frog muscle fibres, NO_3 and Zn^{2+} greatly increase the amplitude and duration of both Ca^{2+} transient and contractile responses while caffeine at 1 mM potentiates twitch amplitude, without affecting the response time course (Caputo and Bolaños, unpublished). In mammalian fibres, Ca^{2+} transients are potentiated in a similar way by lyotropic anions, while the effect of caffeine is much less robust.

With respect to caffeine, and other alkaloids, including ryanodine, it was known that, depending on the concentration, they could potentiate twitches or induce contractures by interacting directly with the RyR (Lüttgau and Oetliker 1968; Weber and Herz 1968). In amphibian skeletal muscle, two isoforms of RyR are expressed in similar amounts, RyR1 and RyR3. While RyR1 is strictly located in the triadic junctional region, RyR3 has also been found in the extrajunctional region. Thus, in frog muscle fibres, due to the alternate disposition of RyR and DHPR, half of the RyR located in the junctional region, plus the amount located extrajunctionally, are not coupled to DHPR, suggesting a different gating mechanism. Evidence has been obtained indicating that caffeine directly acts on the latter channels that serve as target for voltage-independent activation.

In mammalian muscle, RyR3 is only expressed during post-natal development, almost disappearing in adult animals, except for the case of few muscles, like diaphragm and soleus, possibly explaining why caffeine is much less effective than in amphibian muscles. In support of this possibility, it was shown that responses to caffeine decreased during mice post-natal development and in adult animals caffeine remained effective only in those muscles as soleus and diaphragm that contained RyR3 (Rossi et al. 2001).

In conclusion, contractile potentiators have provided valuable insight into the mechanisms pertaining ECC, such as the role of DHPR as the voltage sensors for ECC. More recently, they are helping in understanding the differential functional significance of RyR1 and RyR3.

Role of mitochondria in the ECC mechanism

Mitochondria are organelles present in most cell types, in variable number, depending on the metabolic function and energetic needs of the tissue. The development of new techniques such as electron microscopy 3D reconstructions, tomography, CLSM, targeted mitochondria fluorescent probes (i.e. GFP-fusion proteins), and other molecular biology techniques, have provided copious information on mitochondria structure, organization and relationship with other subcellular organelles such as the SR. In this section, besides presenting recent evidence about muscle mitochondria relationship with the SR, we have also aimed at covering mitochondrial channels and inward Ca^{2+} transport as part of a more complex ECC sequence.

The outer mitochondrial membrane (OMM), although traditionally considered freely permeable, is a critical determinant for the mitochondrial Ca^{2+} accumulation. Nowadays, it is known that Ca^{2+} import across the OMM occurs through a Voltage Dependent Anionic Channel (VDAC), first described by Schein et al. (1976), and later identified as a porin of 30-kDa (Zalman et al. 1980; Mannella et al. 1983; Rizzuto et al. 2009). Increased expression of VDAC enhances Ca^{2+} signal propagation into the mitochondria increasing the extent of mitochondrial Ca^{2+} uptake (Rapizzi et al. 2002).

The inner membrane (IMM) is semi-permeable and highly selective; besides containing all the machinery for oxidative phosphorylation, it also contains Ca^{2+} channels and transporters. The discovery of the electron chain transport and the chemiosmotic hypothesis formulation by Mitchell and Moyle (1967) led to the proposal that Ca^{2+} uptake was mediated by a Ca^{2+} channel, named Mitochondrial Calcium Uniporter (MCU), and driven by the large potential difference (–150 to –180 mV) established by the electrogenic extrusion of protons (Kirichok et al. 2004; Baughman et al. 2011; De Stefani et al. 2011).

MCU is a low affinity/high capacity (Kd of 10–20 μM) oligomeric complex, identified as a highly selective Ca^{2+} channel (Gunter and Pfeiffer 1990; Gunter et al. 1994; Bernardi 1999; Kirichok et al. 2004) formed by a 40-kDa protein with two transmembrane domains (Baughman et al. 2011; De Stefani et al. 2011). It was recently found that MCU of mitoplasts from skeletal muscle present higher conductance than from other tissues (Fieni et al. 2012). At rest, Ca^{2+} concentration in mitochondria and cytoplasm are similar, due to the activity of Ca^{2+} extruding systems and to the low activity of MCU under the normally low cytoplasmic Ca^{2+} conditions (Alonso et al. 2006). Because of its Kd, only when global Ca^{2+} rises above the micromolar level, the MCU is activated and a net uptake of Ca^{2+} by the mitochondria occurs, which is later slowly reversed, until reestablishment of the resting Ca^{2+} (Rizzuto and Pozzan 2006; Bolaños et al. 2009). The establishment of high Ca^{2+} microdomains around the sites of release by IP3R or RyR will favor the Ca^{2+} transport into mitochondria (Rizzuto et al. 1993; Rizzuto and Pozzan 2006; Drago et al. 2012).

A rapid mode of Ca^{2+} uptake (RaM) has been shown to occur in response to imposed Ca^{2+} signals in isolated liver and heart mitochondria (Sparagna et al. 1995; Bernardi 1999; Buntinas et al. 2001). The RaM activates transiently at the beginning of cytoplasmic Ca^{2+} pulses and is detectable above 200 nM in isolated heart mitochondria (Sparagna et al. 1995; Bernardi 1999; Buntinas et al. 2001). An additional Ca^{2+} uptake inhibited by ryanodine indicates the presence of mRyR within the IMM. This channel, which shares several similar biochemical, pharmacological, and physiological properties with both the RyR and RaM (Beutner et al. 2001) have been identified as RyR1 (Beutner et al. 2005). Both uptake modes (RaM and mRyR) exhibit kinetics, Ca^{2+} dependence, and pharmacology that allow them to be distinguished from the MCU (Ryu et al. 2010, 2011).

Two different antiporter systems responsible for exporting or importing Ca^{2+} have been described in mitochondria, the NCX (mNCX) which shares properties with the classical NCX (Jung et al. 1995; Smets et al. 2004) and is expressed in excitable cells, and the H/Ca^{2+} (mHCX) exchanger, present in non-excitable cells (Carafoli et al. 1974; Bernardi 1999; Jiang et al. 2009) and whose molecular identity is the Leucine zipper EF-hand transmembrane protein (Letm1). This protein allows mitochondrial Ca^{2+} uptake at nanomolar concentrations (Jiang et al. 2009).

Finally, the co-existence of low (MCU) and high-affinity (RaM, RyR and Letm1) modes of Ca^{2+} uptake into mitochondria, would allow different mitochondrial populations to take up different amounts of Ca^{2+} during cell activation, thus modulating Ca^{2+} signalling, depending on their location relative to Ca^{2+} stores and channels (Santo-Domingo and Demaurex 2010).

The permeability transient pore (PTP) (Hunter and Haworth 1979) is a large pore whose identity had remained elusive, and spans both IMM and OMM and is activated by Ca^{2+} overloading (Kinnally et al. 1989; Petronilli et al. 1989; Bernardi 1992, 1999; Saris and Carafoli 2005; Zoratti et al. 2005; Bernardi and von Stockum 2012). It can also operate as a Ca^{2+} release channel under physiological conditions (Bernardi and von Stockum 2012). Recently, it has been found that reconstituted dimers of the FoF1 ATP synthase form a channel with properties identical to those of the PTP (Giorgio et al. 2013).

In skeletal muscle, mitochondria occupy 10–15 % of the fibre volume and are mainly located either peripherically in subsarcolemmal clusters or between myofibrils (Fig. 3), largely within the I-bands, surrounding the SR network (Gauthier and Padykula 1966; Eisenberg 1983). This structural arrangement is developmentally regulated, with mitochondria arranged in non-ordered longitudinal fashion in newborn mice (Boncompagni et al. 2009; Rossi et al. 2011). These intermyofibrillar mitochondria are highly organized in pairs at the I-band level, close to the CRU on either side of the Z-line, between T-Tubules, and contacting the SR near sites of Ca^{2+} uptake by the SERCA (Ogata and Yamasaki 1985; Ramesh et al. 1998; Vendelin et al. 2005; Franzini-Armstrong 2007; Bolaños et al. 2008). This arrangement favours fast ATP delivery to support Ca^{2+} transport into SR and to participate in Ca^{2+} homeostasis capturing Ca^{2+} from high Ca^{2+} microdomains near the sites of release (Rizzuto and Pozzan 2006; Shkryl and Shirokova 2006; Franzini-Armstrong 2007; Rossi et al. 2011; Yi et al. 2011; Drago et al. 2012). Subsarcolemmal mitochondria are organized in clusters, densely packed and less regularly arranged as compared with intermyofibrillar ones (Ogata and Yamasaki 1985; Kuznetsov et al. 2006). Skeletal muscle fibre types show differences in their mitochondria content being higher in slow-twitch fibres (Gauthier and Padykula 1966; Ogata and Yamasaki 1985), as well as morphological and functional diversity, existing specialisation in function between mitochondria from both slow-oxidative and fast-glycolitic fibre types (Kuznetsov et al. 2006; Picard et al. 2012).

The close proximity of mitochondria to the SR and calcium release units has been studied in many preparations using many techniques (Franke and Kartenbeck 1971; Morre et al. 1971; Lewis and Tata 1973; Shore and Tata 1977; Meier et al.

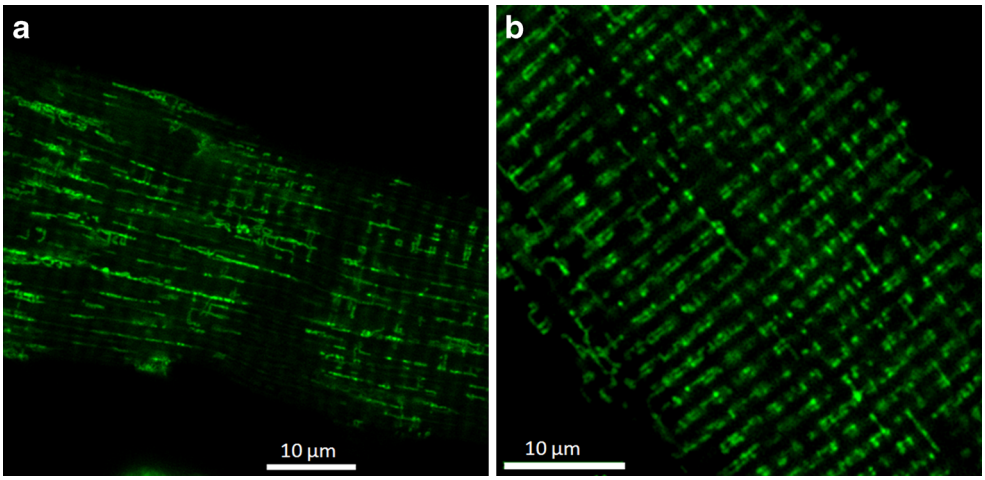

Fig. 3 Subsarcolemmal (*left*) and inner (*right*) differential mitochondrial distribution in flexor digitorum brevis muscle fibres stained with Mitotracker Green. The images were acquired with a Nikon C1 confocal microscope. A pattern of paired columns of mitochondria, parallel to the short axis of the cell, can be identified in the inner or intermyofibrillar location, while single, longer rows of mitochondria, parallel to the long axis of the cell, can be identified in the subsarcolemmal region of the cell

1981; Mannella et al. 1998; Franzini-Armstrong and Boncompagni 2011). It was shown that as much as 20 % of the mitochondrial surface is in direct contact with the ER (Rizzuto et al. 1998) and that the maintenance of a proper spacing between ER and mitochondria seems to guarantee cell function and survival in some tissues (Csordás et al. 2006). In mouse FDB muscle fibres, strands of 3–4 nm diameter and 9–10 nm length (Franzini-Armstrong 2007; Boncompagni et al. 2009) appear to anchor mitochondria to the SR. The number of these tethers increases during postnatal development and might restrict mitochondrial movement away from sites of SR Ca^{2+} release, especially during contractile movements and shortening (Boncompagni et al. 2009; Rossi et al. 2011), providing a structural framework for bidirectional SR-mitochondrial signalling (Dirksen 2009b). In adult skeletal muscle, since mitochondria are anchored on the far side of the triads a diffusional distance of about ~130 nm separates the release sites from mitochondria (Franzini-Armstrong 2007), and a dissipation of Ca^{2+} microdomains could occur (Stern 1992). However, in developing muscle, this distance can be less than 100 nm and mitochondria could experience a significant Ca^{2+} release microdomain (Rossi et al. 2011).

Although the exact nature of tethers remains unresolved, it has been proposed that they are formed by a complex termed the "ER–mitochondria encounter complex" (ERMES), involving several proteins present in the OMM and in the ER that appear essential to keep the contact points between the two organelles in yeast (Kornmann et al. 2009). In mammals, silencing mitofusin disrupts ER morphology and loosens ER–mitochondria interactions, thereby reducing the efficiency of mitochondrial Ca^{2+} uptake in response to stimuli (de Brito and Scorrano 2008; see Eisner et al. 2013).

Mitochondrial Ca^{2+} uptake has been shown to occur during electrically elicited contractile responses in mouse muscle fibres, with a relatively short delay, 10 ms, between the Ca^{2+} signal in the myoplasm and that in the mitochondria (Rudolf et al. 2004; Yi et al. 2011). Transgenic mice expressing the Ca^{2+}-sensitive bioluminescent reporter GFP-aequorin targeted to the mitochondrial matrix were studied in mice in vivo and showed a readily detected rapid Ca^{2+} rise inside the mitochondrial matrix during single-twitch muscle contractions (Rogers et al. 2007). In both slow- and fast-twitch fibres from rat, mitochondria are capable of accumulating Ca^{2+} in the presence of BAPTA that suppress Ca^{2+} transients but not mitochondrial Ca^{2+} increases, suggesting some kind of Ca^{2+} tunnelling from SR to mitochondria (Shkryl and Shirokova 2006).

In muscle cell lines and myotubes, mitochondria can pick up Ca^{2+} mobilised from a single RyR release unit (sparks) leading to the generation of single mitochondrial miniature Ca_{myt} signals (Ca^{2+} marks) and to feedback control on the Ca^{2+} release (Pacher et al. 2002; Isaeva et al. 2005).

Inhibition of mitochondrial Ca^{2+} uptake, either by blocking MCU with ruthenium red or by dissipating the mitochondrial membrane potential with protonophores like Carbonyl cyanide 4-trifluoromethoxy phenylhydrazone (FCCP), results in a large cytoplasmic Ca^{2+} increase with a slow rate of recovery (Rizzuto et al. 2000; Caputo and Bolaños 2008). In mice FDB muscle fibres, poisoning the mitochondria with FCCP (Fig. 4) causes a sizeable and rapid increase in the basal Ca^{2+} concentration followed by a marked decrease of Mag-Fluo-4 Ca^{2+} transients, and variable effects on the time course of Ca^{2+} transient decay, probably related to the composition of fibre types in FDB fibres (Caputo and Bolaños 2008; Calderón et al. 2009). Ca^{2+} uptake by mitochondria has also been observed in mice FDB fibres loaded with Rhod-2 and subjected to a SR depletion procedure to study SOCE (Fig. 5) or Ca^{2+} release in FDB fibres loaded with CaOrange-5N and exposed to the protonophore FCCP (Bolaños et al. 2008, 2009).

Ca^{2+} mitochondria uptake after contractile activation during repetitive tetanic stimulation in toad and mouse fibres is reduced by FCCP poisoning (Lännergren et al. 2001; Bruton et al. 2003). In PV knockout mouse, the muscle fibres content of mitochondria doubles and the fibres appear to have an increased resistance to fatigue. The increased mitochondrial

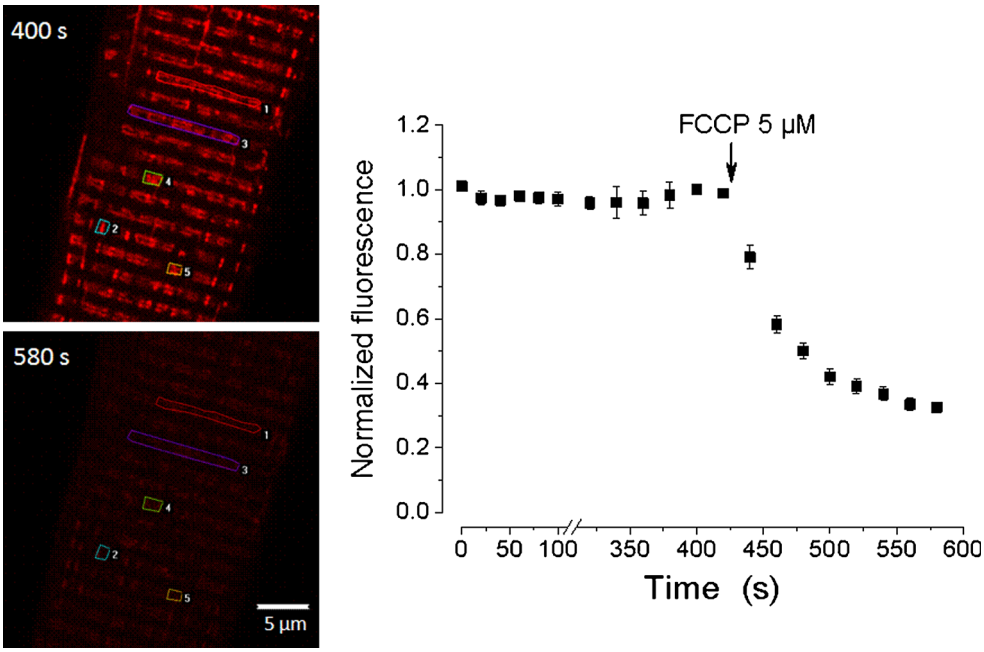

Fig. 4 Time course of fluorescence decay after FCCP poisoning (*right*), in one flexor digitorum brevis fibre stained with TMRE to visualize mitochondrial potential (Ψm). On the *left*, confocal images of the cell indicating regions of interest (ROI, *numbered squares*) and time of acquisition after starting the experiment are shown. On the *right*, the *black squares* represent the mean±SEM of the measurements carried out at level of the ROI as a function of time. A rapid decrease in fluorescence, suggesting a dissipation of Ψm, is seen after the application of FCCP

content results in a faster than expected removal of Ca^{2+} following brief tetanic stimulation (Racay et al. 2006).

Even when it is clear that mitochondria have a role in Ca^{2+} regulation during ECC and during contractile activity, their physiological relevance for different fibre types is not clear. Some have claimed that mitochondria may be relevant mainly in slow-twitch fibres, but others have not seen differential effect of blocking mitochondrial Ca^{2+} uptake in slow- and fast-twitch fibres during a tetani (Gillis 1997; Sembrowich et al. 1985; Lännergren et al. 2001; Calderón et al., unpublished results).

Future work may address the details of the SR-mitocondrial communication in skeletal muscle, and the tethers nature, considering also different fibre types, differences between subsets of mitochondria in skeletal muscle and different conditions such as fatigue, ageing and diseases.

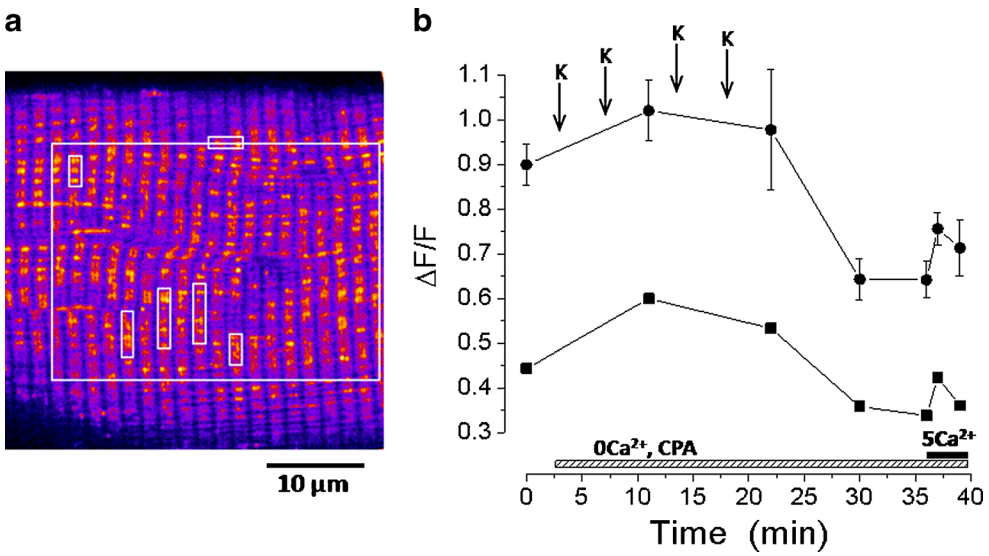

Fig. 5 Increase in intramitochondrial Ca^{2+} during a sarcoplasmic reticulum (SR) depletion protocol in a flexor digitorum brevis fibre imaged in a confocal microscope. **a** A pseudocolor image of mitochondria loaded with Rhod-2. **b** Summarizes the time course of the mitochondrial mean Rhod-2 fluorescence variation in the regions of interest (*white squares*) marked in (**a**). The *black circles* represent the mean fluorescence in the *small white squares* in (**a**), and the *black squares* the mean fluorescence in the *big white square* in (**a**). The mitochondrial fluorescence increases during the protocol in which the cell is in absence of external Ca^{2+} and the SR Ca^{2+} ATPase is blocked by cyclopiazonic acid (CPA). When 5 mM Ca^{2+} external solution reaches the cell the mitochondria uptake part of the Ca^{2+} entering the fibre via the store operated Ca^{2+} entry mechanism. *K* indicates the moments in which an external solution with high K^+ concentration was applied to elicit SR Ca^{2+} release in order to deplete this Ca^{2+} store

SOCE-mitochondria-ECC relationship

SOCE was originally described in non-excitable cells, where it constitutes a major pathway for Ca^{2+} influx. It was denominated capacitative calcium influx (I_{CRAC}) and characterised as a very small but highly Ca^{2+} selective current that insures the replenishment of the ER (Putney 1986; Hoth and Penner 1992; Parekh and Putney 2005).

SOCE has also been demonstrated in excitable cells (Bernardi 1999; Rizzuto et al. 2000; Parekh and Putney 2005) as adult skeletal muscle fibres (Kurebayashi and Ogawa 2001; Ma and Pan 2003; Ducret et al. 2006; Gonzalez Narvaez and Castillo 2007; Launikonis and Ríos 2007; Bolaños et al. 2009; Dirksen 2009a), though there is less information on the regulatory mechanisms involved in SOCE activation in excitable cells, in particular adult skeletal muscle fibres, than in non-excitable cells.

The molecules involved in the SOCE complex have been identified. The first one, the stromal interacting molecule STIM1, present in the ER/SR membrane, senses the degree of filling of Ca^{2+} in the ER/SR (Liou et al. 2005; Roos et al. 2005; Zhang et al. 2005). The second, Orai1, has been identified as the conductive pore sub-unit of the I_{CRAC} channel (Feske et al. 2006; Prakriya et al. 2006; Vig et al. 2006). In mammals, two STIM genes and three Orai genes have been identified (Zhang et al. 2005; Feske et al. 2006; Vig et al. 2006). When Ca^{2+} levels are low, STIM1 clusters in regions of the ER/plasma membrane junctions denominated punctae, where it interacts with Orai1 activating Ca^{2+}-influx (Luik et al. 2006; Smyth et al. 2006; Soboloff et al. 2006; Muik et al. 2009). In endothelial cells exposed to FCCP and/or oligomicin, STIM1–Orai1-dependent SOCE is completely prevented, thus pointing to an essential contribution of mitochondrial Ca^{2+} handling to STIM1–Orai1-dependent SOCE. These finding suggest that a Ca^{2+}-dependent process in the mitochondria unlike (or in addition to) local Ca^{2+} buffering is essential and specific for the activity of the STIM1–Orai1-dependent SOCE (Naghdi et al. 2010).

In skeletal muscle, STIM1 and Orai1 are highly expressed (Stiber et al. 2008; Vig et al. 2008) and are localized at triadic level and in the SR terminal cisternae. In this tissue, SOCE activation and deactivation is in the order of milliseconds indicating that STIM1 and Orai1 must be evenly distributed throughout the junctional membrane and can activate rapidly (Launikonis and Ríos 2007; Stiber et al. 2008; Edwards et al. 2010). Two models that allow for rapid SOCE activation upon SR Ca^{2+}-store depletion were proposed by Dirksen (2009a). The first one states that STIM1 monomers are prelocalized at SR terminal cisternae in the vicinity of inactive Orai1 channels at the triadic junctions. When Ca^{2+} dissociates from STIM1, in response to SR depletion, their conformational change and oligomerization will permit the activation of Orai1 also prelocalized in the T-tubule membrane. The second model proposes that STIM1/Orai1 complex exists preformed but inactive until SR Ca^{2+} depletion reaches a certain level, triggering direct activation of Orai1-mediated Ca^{2+} influx. This last possibility would allow an ultrafast, very efficient and controlled activation of Ca^{2+} influx through Orai1 (Launikonis and Ríos 2007; Dirksen 2009a; Edwards et al. 2010).

Though it seems that SOCE has no physiological role in skeletal muscle short-term activation, and it is not required to refill SR (Cully and Launikonis 2013) Ca^{2+} entry through SOC is crucial for long-term Ca^{2+} homeostasis, such that reduced SOC activity exaggerates muscle fatigue under conditions of intensive exercise (Pan et al. 2002).

In FDB adult mouse skeletal muscle fibres, FCCP mitochondria poisoning increases the Ca^{2+} myoplasmic levels, reduces the amplitude of Ca^{2+} transients and reduces SOCE (Fig. 6) (Bolaños et al. 2008, 2009; Caputo and Bolaños 2008). This effect has been explained in terms of different mechanisms: (1) reduction of Ca^{2+} uptake by mitochondria increases cytoplasmic Ca^{2+}, thus favouring Ca^{2+} dependent inactivation of the SOC channels (Hoth et al. 2000); (2) removal of mitochondrial competition with SERCA pumps that favors store replenishment (Parekh 2003); and (3) inhibition of some factor produced by functional mitochondria necessary for SOCE activation (Glitsch et al. 2002; Naghdi et al. 2010). The close functional relationship between SR and mitochondria (Isaeva and Shirokova 2003; Shkryl and Shirokova 2006; Bolaños et al. 2008; Boncompagni et al. 2009; Rossi et al. 2011) facilitates the establishment of microdomains (Rizzuto et al. 1993; Berridge 2006; Rizzuto and Pozzan 2006) and the uptake of Ca^{2+} by mitochondria immediately after its release from the SR or the influx through SOCs, before it can diffuse out the restricted space of the SR–mitochondrial junctions.

In spite of recent studies claiming that SOCE is not relevant in skeletal muscle for SR Ca^{2+} replenishment (Cully and Launikonis 2013), all the machinery needed to activate it is present at the triad. On the other hand, it is known that SOCE activation requires fully energized mitochondria in excitable and non-excitable tissues (Glitsch et al. 2002; Bolaños et al. 2009; Naghdi et al. 2010). Future work must be addressed to clarify not only the physiological relevance of SOCE but also its modulation by mitochondria and/or other components involved in ECC in skeletal muscle.

Ca^{2+} sparks

Ca^{2+} sparks were first described as minute, spontaneous fluorescence signals originating from highly restricted zones of cardiac myocytes (Cheng et al. 1993). They could also be elicited, at a much higher frequency, by cell membrane depolarization. The occurrence of the events did not depend on external Ca^{2+} or on Ca^{2+} entry through the L-type Ca^{2+} channels. The properties of the sparks, and the localization

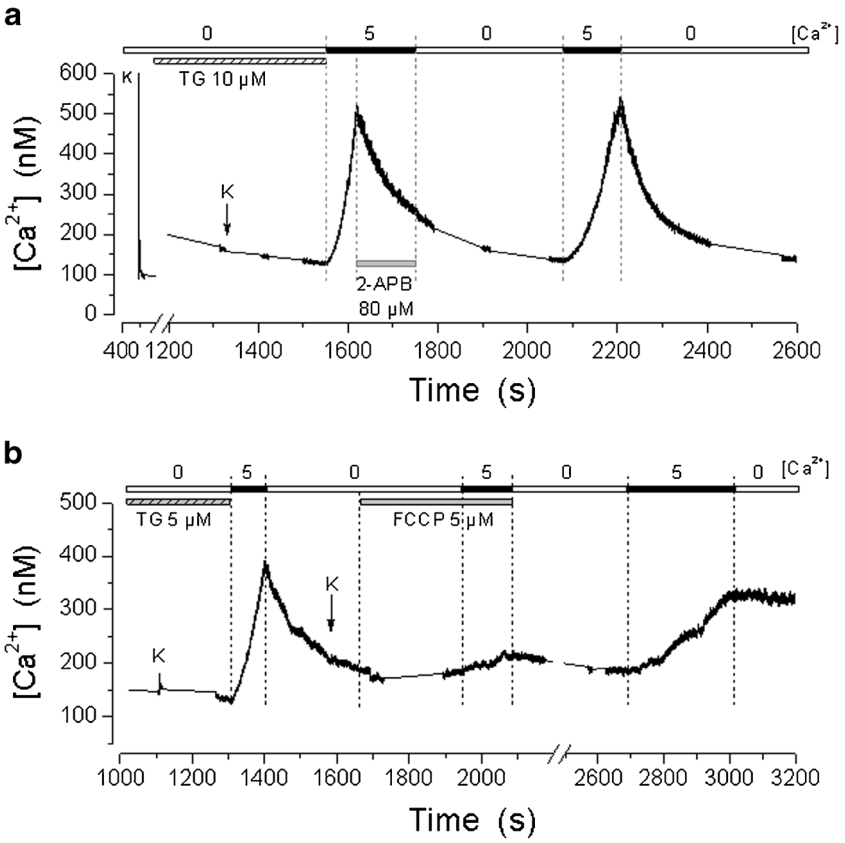

Fig. 6 Activation of store-operated Ca^{2+} entry (SOCE) in a flexor digitorum brevis fibre loaded with Fura-2 after sarcoplasmic reticulum (SR) depletion by high K^+ exposures in the absence of external Ca^{2+} and in the presence of thapsigargin (TG) 5-10 µM. **a** Once depleted as shown by the absence of response to high K^+, Ca^{2+} reintroduction in the external medium activates SOCE. The Ca^{2+} entrance can be reversibly blocked by 80 µM 2-APB. **b** When a similar protocol is applied to another fibre, but exposed to FCCP, SOCE cannot be activated again. It can be partially recovered after a long washout of the drug. This suggests that the mitochondrial depolarization affects the Ca^{2+} entry induced by SR depletion

of their origin, at the level of the junctional region between SR and T-tubules, led to the conclusion that they derived from point Ca^{2+} sources, and constituted the elementary Ca^{2+} release events for ECC, resulting from the transient openings of Ca^{2+} release channels. In the heart, these channels were identified as the RyR2, activated via a CICR mechanism, by Ca^{2+} entering the myocytes through the DHPR (Cheng and Lederer 2008).

Ca^{2+} sparks were also demonstrated in amphibian striated muscle fibres (Tsugorka et al. 1995), where they could occur spontaneously or be evoked by membrane depolarization; in both cases, their spatio-temporal characteristics were similar and were not affected by the level of the membrane potential in the range from –80 and +40 mV (LaCampagne et al. 1996). The frequency of spark generation was potential-dependent and could be modulated by Ca^{2+} that increased it and Mg^{2+} that had the opposite effect (Zhou et al. 2004).

Contrary to the initial idea that one spark corresponded to the opening of a single release channel, Shtifman et al. (2000) estimated the number of release channels contributing to the generation of one event to be between 2 and 4. Moreover, the idea that one spark represented one quantum of Ca^{2+} release was also challenged by the demonstration of diffused Ca^{2+} release, not associated with defined sparks, both in cardiac (Lipp and Niggli 1996) and in skeletal (Shirokova and Ríos 1997) muscle fibres.

Also in skeletal muscle, Ca^{2+} sparks occur at level of the triadic junctional region (see "The excitation–contraction coupling (ECC) mechanism in skeletal muscle"). Due to the alternate disposition of the RyR and DHPR arrays, for each tetrameric molecule facing, and under control of, a tetrad, there is one molecule that is not. Furthermore, the presence of another RyR isoform, RyR3, that forms arrays in the extra-junctional regions of amphibian skeletal muscle fibres, increases the fraction of Ca^{2+} release channels that are not under control of the DHPR (Felder and Franzini-Armstrong 2002).

Opposite to the case of frog muscles, Ca^{2+} sparks are rarely observed in mammalian muscle under physiological conditions. Instead, release events not composed of Ca^{2+} sparks were observed in response to membrane depolarization or exposure to caffeine leading to the proposal that in rats this release was the unique response to voltage activation, while in frog the initial release events served as a trigger for CICR responses visualised as sparks. The idea that RyR3 was associated with spark generation in amphibian was proved correct by the demonstration that exogenous expression of RyR3 in adult mouse muscle caused an abundance of sparks in response to depolarisation, changing a spark-free mammalian response, into a frog one (Pouvreau et al. 2007b).

On the other hand, in mammalian muscle, Ca^{2+} sparks could be observed under special conditions, such as permeabilization by saponine, osmotic stress, membrane damage

and mitochondrial metabolic uncoupling (Apostol et al. 2009; Weisleder 2012; Isaeva and Shirokova 2003), demonstrating that the machinery and capacity for spark generation are present in mammalian muscle, but somehow repressed. In favour of this view, it has been shown that, during myotubes differentiation, the presence of T-tubules determine the absence of Ca^{2+} sparks and of RyR1 and RyR3, that abound where T-tubules are not present (Zhou et al. 2006).

Although the specific mechanisms by which RyR1 and RyR3 operate have not yet been clarified, there is strong evidence pointing to the importance of RyR3 in determining the mode of Ca^{2+} release.

Perspectives

Some tasks still remain to gain full knowledge on ECC. The search for new minor ECC proteins (Treves et al. 2009) and to study their role in fatigue, ageing and diseases, as well as their variability according to fibre types, should continue. The application of ECC knowledge is of paramount importance to fight muscle diseases and help in conditions such as ageing (Wang et al. 2012; Mosca et al. 2013). To develop a mathematical model on ECC that integrates most of the recent information gathered on ECC under different conditions will allow us to better understand and manipulate Ca^{2+} kinetics. Also, a better understanding of the relationsip between ECC machinery and muscle biochemical and metabolic functions (besides the production of ATP) is an important endeavor that deserves full attention in the coming years. Considering that many RyR are not directly coupled to DHPR, and that CICR is not operational in mammalian muscle under physiological conditions, the puzzle that remains to be solved is whether and how the DHPR-coupled RyR signal the uncoupled ones to synchronize Ca^{2+} release. In addressing many of these questions it is likely that the use of super-resolution microscopy and the improvement of its time-resolution will allow for a new era of physiological studies.

Conclusions

In conclusion, the ECC mechanism represents a rapid communication between electrical events occurring in the plasma membrane and the Ca^{2+} release from the SR, which leads to skeletal muscle contraction. A large body of knowledge on the concerted function of the main macromolecules and some minor molecules involved in ECC mechanism has been gathered as a result of six decades of research. Additionally, ECC understanding will benefit from the continuous gain in temporal and spatial resolution of most of the techniques currently used for ECC study. The application of the knowledge gathered on ECC will likely help understand the pathophysiology of some muscle diseases and develop tools to fight them.

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Conflict of interest Juan C. Calderón, Pura Bolaños and Carlo Caputo declare that they have no conflict of interest.

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