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Voltage clamp methods for the study of membrane currents and SR Ca²⁺ release in adult skeletal muscle fibres

Erick O. Hernández-Ochoa[#] and Martin F. Schneider^{#,*}

[#]Department of Biochemistry and Molecular Biology, University of Maryland School of Medicine, 108 N. Greene St., Baltimore, Maryland, 21201

Abstract

Skeletal muscle excitation-contraction (E-C)¹ coupling is a process composed of multiple sequential stages, by which an action potential triggers sarcoplasmic reticulum (SR)² Ca²⁺ release and subsequent contractile activation. The various steps in the E-C coupling process in skeletal muscle can be studied using different techniques. The simultaneous recordings of sarcolemmal electrical signals and the accompanying elevation in myoplasmic Ca²⁺, due to depolarization-initiated SR Ca²⁺ release in skeletal muscle fibres, have been useful to obtain a better understanding of muscle function. In studying the origin and mechanism of voltage dependency of E-C coupling a variety of different techniques have been used to control the voltage in adult skeletal fibres. Pioneering work in muscles isolated from amphibians or crustaceans used microelectrodes or 'high resistance gap' techniques to manipulate the voltage in the muscle fibres. The development of the patch clamp technique and its variant, the whole-cell clamp configuration that facilitates the manipulation of the intracellular environment, allowed the use of the voltage clamp techniques in different cell types, including skeletal muscle fibres. The aim of this article is to present an historical perspective of the voltage clamp methods used to study skeletal muscle E-C coupling as well as to describe the current status of using the whole-cell patch clamp technique in studies in which the electrical and Ca²⁺ signalling properties of mouse skeletal muscle membranes are being investigated.

Keywords

skeletal muscle; excitation; contraction; charge movement; Ca²⁺ current; Ca²⁺ transients; voltage clamp; patch clamp; whole-cell configuration

1. Introduction

Numerous cellular processes, including ionic transport and enzyme and membrane receptor activity, are influenced by the cell membrane potential (Armstrong, 1981; Bezanilla, 2008).

¹excitation-contraction (E-C)

²sarcoplasmic reticulum (SR)

*To whom all correspondence should be addressed: Martin F. Schneider, 108 N. Greene St., Baltimore, MD, USA, 21201. Department of Biochemistry and Molecular Biology, University of Maryland School of Medicine. Telephone: 1+(410) 706-7812, mschneid@umaryland.edu.

Conflict of Interest

We declare no competing financial interests.

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The coordinated activity of these processes in turn regulates more complex cellular functions such as excitability, secretion, muscular contraction, metabolism and gene expression (Armstrong and Hille, 1998). Skeletal muscle excitation-contraction (E-C) coupling is a process, composed of multiple sequential stages, by which an action potential (AP)³, a sudden transient depolarizing change in membrane potential, triggers sarcoplasmic reticulum (SR) Ca²⁺ release and subsequent contractile activation (Beam and Horowicz, 2004; Sandow, 1952; Schneider, 1994). Depolarization of the transverse tubule (TT)⁴ system is detected by the voltage sensor of E-C coupling, the dihydropyridine receptor (DHPR)⁵, a Cav1.1 L-type Ca²⁺ channel activated by voltage (Rios and Brum, 1987; Rios and Pizarro, 1991; Schneider, 1994; Schneider and Chandler, 1973). Upon depolarization, the voltage sensing apparatus of the Cav1.1 is used as a trigger for Ca²⁺ release from SR by means of molecular coupling of Cav1.1 to the immediately apposed ryanodine receptor/SR Ca²⁺ release channels, which can deliver far more calcium than can enter through the Cav1.1 Ca²⁺ channels themselves (Catterall, 1991; Fill and Copello, 2002; Rios and Pizarro, 1991; Schneider, 1994). The released Ca²⁺ binds to myosin-actin contractile machinery and produces force or shortening. When Ca²⁺ release terminates, Ca²⁺ is returned to the SR via the ATP-dependent Ca²⁺ pump in the SR membrane, causing fibre relaxation and restoring the initial resting state of the fibre (Beam and Horowicz, 2004; Berchtold et al., 2000; Caputo, 2001; Melzer et al., 1995).

2. Molecular determinants of membrane excitability and voltage sensing in skeletal muscle

2.1 Voltage dependent channels and the muscle action potential

The principal duty performed by the action potential in a skeletal muscle fibre involved in locomotion is the activation of the contractile machinery in response to cortical voluntary commands delivered via motor neuron pathways, and conveyed at the neuromuscular junction via endplate potentials that generate the muscle action potentials. The AP then propagates longitudinally along the sarcolemma as well as radially into the muscle fibre TT-system (Bezaniilla et al., 1972; González-Serratos, 1971; Huxley and Taylor, 1958) (Fig. 1A). The depolarizing phase of the muscle action potential is mediated by the opening of voltage-dependent Na⁺ channels (Nav1.4). Repolarization is due in part to the inactivation of Na⁺ channels and to the opening of both voltage dependent and voltage-independent K⁺ channels (i.e., Kv1.4, Kv3.4, Kv7.5, Kir2.1, Kir6.1, KCa1.1) and voltage-dependent Cl channels (ClC1 and ClC2), with minimal influence of voltage-dependent Ca²⁺ channels (Cav1.1) (Jurkat-Rott et al., 2006; Wolters et al., 1994). The afterdepolarization of a skeletal muscle fibre is composed of two phases, an early one considered to be caused by spreading of the spike into the TT-system and a later one due to an accumulation of potassium ions in the TT-system, which increases with repetitive APs (Almers, 1980; Kirsch et al., 1977). As in the case of different excitable cells, dozens of different ion channels and ion transporters (i.e., ATP1A1, ATP2, and multiple ion exchangers) determine the excitability properties of the skeletal muscle fibre. For more details on the diversity and expression of ion channels and ion transporters of skeletal muscle see the review by Jurkat-Rott and Lehmann-Horn (Jurkat-Rott et al., 2006).

2.2 Cav1.1: transverse tubule voltage sensor for excitation contraction coupling

Depolarization of the TT-system is detected by the voltage sensor of E-C coupling (Fig. 1A–B), classically referred to as the DHPR, a calcium channel (L-type or Ca_v1.1) activated by

³action potential (AP)

⁴transverse tubule (TT)

⁵dihydropyridine receptor (DHPR)

voltage (Rios and Pizarro, 1991; Schneider and Chandler, 1973). Cav1.1 is predominantly expressed in the membrane of the TT-system of adult skeletal muscle and is a member of the diverse family of voltage-dependent Ca^{2+} channels. Voltage operated calcium channels in vertebrate skeletal muscle have evolved to serve a highly specialized role. Like its counterparts in other cell types such as cardiac (Cav1.2) and neuronal (Cav1.4) cells, Cav1.1 channels pass Ca^{2+} current into the muscle cell when opened by an action potential. However, the Cav1.1 Ca^{2+} current is not enough to activate muscle's contractile proteins (Sanchez and Stefani, 1978; Schwartz et al., 1985). In fact, the macroscopic current generated by Cav1.1 is comparable in amplitude to its channel gating current (Delbono, 1992; Prosser et al., 2009b; Wang et al., 1999). Instead of primarily generating Ca^{2+} current, the essential function of Cav1.1, which is a membrane spanning protein located in the surface and TT- membranes of the muscle (Fig. 1B–C), results from its direct interaction with the ryanodine receptor type-1 (RyR1), the homotetrameric non-voltage-dependent Ca^{2+} release channels located inside the muscle fibre in the membrane of the junctional SR (Fig. 1B) (Beam and Horowicz, 2004; Schneider, 1994). This process occurs at very specialized “triad” junctions between the TT system and the SR.

Cav1.1 is a heteromultimeric integral membrane protein composed of five subunits, alpha1, alpha2, beta, gamma and delta (Fig. 1C). The alpha1 subunit is a protein of about 2000 amino acid residues in length with an amino acid sequence and structure similar to that of the voltage-gated sodium channels (Catterall, 2000; Catterall, 2011; Tanabe et al., 1987). The amino acid sequence is organized in four repeated domains (I–IV), each containing six transmembrane segments (S1–S6), connected by both intracellular and extracellular loops (Fig. 1C). The alpha subunit contains the ion-conducting pore (a membrane associated loop between S5 and S6 (Catterall, 2000; Catterall, 2011). The alpha1 subunit determines the major characteristics of the cation channel complex such as ion selectivity, permeability, voltage sensitivity (via the S1–S4 voltage sensing module) intracellular signalling modulation and pharmacological regulation. The S4 segments and perhaps other charged residues of each homologous domain serve as the voltage sensors for activation (Fig. 1C) (Bezanilla, 2008; Catterall, 2000; Catterall, 2011), presumably moving outward and possibly rotating under the influence of the electric field (i.e., measured as non-linear capacitive currents or macroscopic gating currents using voltage clamp techniques) (Rios and Pizarro, 1991; Schneider, 1994; Schneider and Chandler, 1973). The resulting conformational change activates two separate processes: calcium influx via Cav1.1 (Catterall, 1991) and Ca^{2+} release via RyR1 (Rios and Brum, 1987). In the first process, the voltage sensor movement and subsequent intramolecular conformational changes are thought to lead to the opening of the Cav1.1 alpha1 pore (i.e., measured as Ca^{2+} currents using voltage clamp techniques) (Hagiwara et al., 1969; Sanchez and Stefani, 1978; Stanfield, 1977). The opening of Cav1.1 channels in the surface and transverse tubules mediates a relatively slow Ca^{2+} current that may contribute minimally to increasing myoplasmic Ca^{2+} concentration (Melzer et al., 1995) and perhaps to regulating the force of contraction in response to high-frequency trains of nerve impulses (Catterall, 1991; Catterall, 2011). In the second process, the voltage sensor movement and subsequent intra and intermolecular conformational changes are thought to directly activate RyR1 Ca^{2+} release (i.e., measured as myoplasmic $[\text{Ca}^{2+}]$ changes using Ca^{2+} indicators and optical techniques) (Baylor et al., 1983; Kovacs et al., 1979; Miledi et al., 1977). The voltage-driven conformational changes in the voltage-sensing domains of Cav1.1 are thought to directly induce the activation of RyR1 via protein-protein interactions between the Cav1.1 cytoplasmic loop (“II-III loop”) between Cav1.1 alpha 1 domains II and III and the RyR1 (Nakai et al., 1998a; Nakai et al., 1998b; Protasi et al., 2002; Tanabe et al., 1990). In addition, the DHPR beta subunit appears to play a role in signal transmission from Cav1.1 to RyR1 (Beam and Horowicz, 2004).

Two potential and unexplored roles of the skeletal Cav1.1 Ca^{2+} current include possible maintenance of the Ca^{2+} homeostasis and the activation of Ca^{2+} -dependent signalling molecules (i.e., Ca^{2+} -dependent channels or transcription factors) via local Ca^{2+} microdomains.

3. Early (pre-voltage clamp) studies of E-C coupling in adult intact fibres

The study of E-C coupling in skeletal muscle can be approached at different steps (i.e., action potential generation and propagation, sarcolemma and transverse tubule depolarization, voltage sensor activation, Ca^{2+} release and reuptake, contraction and force development) using a diverse array of methods and techniques. Early work on skeletal muscle conducted in the 1950s used microelectrodes and force transducers to measure the action potential and muscle tension (Kahn and Sandow, 1950). Around the same time period, Heilbrunn and Wiercinski (Heilbrunn and Wiercinski, 1947), Hasselbach (Hasselbach, 1964) and Weber (Weber, 1959) pointed out the importance of myoplasmic Ca^{2+} for muscle activity. In 1960, Hodgkin and Horowicz provided the first demonstration of the steep membrane potential dependence of both the activation of skeletal muscle contraction and its subsequent inactivation (Hodgkin and Horowicz, 1960a; Hodgkin and Horowicz, 1960B). In parallel, the electron microscopic studies of Francini-Armstrong and Porter (Francini-Armstrong and Porter, 1964) and Peachey (Peachey, 1965), provided the structural bases of the membrane systems carrying out E-C coupling. Then, during the 1970's voltage clamp techniques were applied to the study of skeletal muscle E-C coupling (Adrian et al., 1970; Schneider and Chandler, 1973).

4. What is voltage clamp?

Intuitively, the concept of voltage clamp is simple. First, we need to measure the membrane potential in the cell under study. We then compare that measurement to the desired ("command") value, and if there is a difference, inject current in the direction appropriate to drive the membrane potential to the command potential (Jones, 1990). In the event that a current is generated in the voltage clamped membrane, the voltage clamp circuitry must pass a current equal and opposite to the membrane current in order to maintain the membrane potential constant. The current passed by the voltage clamp amplifier is the current that happens to be active in the membrane at any given time (Jones, 1990). This capability to measure the membrane current during an imposed voltage wave form (usually a step change in voltage) is the pivotal advantage of the voltage clamp. However, in order to effectively utilize the voltage clamp system, the voltage must be spatially uniform over the membrane area from which the current is recorded. This "space clamp" requirement for effective use of the voltage clamp in characterizing membrane currents was achieved in muscle fibres by using specialized recording configurations and/or conditions as considered below.

5. Combined voltage clamp and optical methods for studying E-C coupling

By end of 1960s Hodgkin's, Hagiwara's and Rougier's laboratories, pioneered the use of voltage clamp techniques in skeletal muscle fibres (Adrian et al., 1966; Adrian et al., 1970; Hagiwara et al., 1969; Hagiwara et al., 1968; Ildefonse and Rougier, 1972; Rougier et al., 1968). In 1973, Schneider and Chandler discovered the intramembrane charge movement (Schneider and Chandler, 1973), which represent the electrical manifestation of molecular charge redistribution, occurring at the level of voltage sensors localized in the sarcolemma and TT. The use of Ca^{2+} sensitive photoproteins (Ashley and Ridgway, 1970) and Ca^{2+} sensitive indicator dyes allowed the recording of global Ca^{2+} transients associated with contractile activation in skeletal muscle (Miledi et al., 1977). These early studies provided the pavement for many later reports, in which non-linear capacitive currents and Ca^{2+}

release were measured simultaneously or in parallel (Baylor et al., 1983; Chandler et al., 1976; Delbono, 1992; Delbono and Stefani, 1993B; Dulhunty and Gage, 1983; Hollingworth and Marshall, 1981; Horowicz and Schneider, 1981a; Horowicz and Schneider, 1981b; Jacquemond et al., 1991; Kovacs et al., 1979; Lamb, 1986a; Melzer et al., 1984; Melzer et al., 1986; Shirokova et al., 1996; Szentesi et al., 1997). These pioneering studies revealed a close physiological correlation between charge movement, Ca^{2+} release and contractile activation. Therefore, the measurement of intramembrane charge movement (experimentally measured in voltage clamp recording conditions as a non-linear capacitive current, a form of macroscopic gating current arising predominantly from Cav1.1 voltage sensor movements), Ca^{2+} currents (measured as an ionic non-linear Ca^{2+} current in a voltage clamped fibre) and myoplasmic Ca^{2+} transients (empirically estimated using optical methods and Ca^{2+} indicators, see below) have proved to be essential for understanding how membrane depolarization is linked to SR Ca^{2+} release in the early and intermediate steps of skeletal E-C coupling.

Based on the preceding considerations, characterization of the control mechanisms for SR Ca^{2+} release in single muscle fibre preparations requires the use of at least two types of measuring methods in order to monitor events occurring at both the TT voltage sensors and at the SR Ca^{2+} release channels (Schneider, 1994). Movements of the TT voltage sensors are monitored as intramembrane non-linear capacitive currents, and charge movement is estimated from these measurements. Such intramembrane non-linear capacitive currents are detected with voltage clamp techniques and appropriate procedures for eliminating the larger ionic and linear capacitive currents flowing through the surface and TT membranes (Rios and Pizarro, 1991; Schneider and Chandler, 1973). In contrast, the SR constitutes an internal membrane system electrically isolated from fibre external membranes. Consequently, no current directly generated by the SR Ca^{2+} release channels is detected by the voltage clamp system. Therefore, SR Ca^{2+} release must be measured using a second monitoring system. This can be achieved either indirectly by measuring fibre contraction (Caputo and Dipolo, 1978; Caputo and Fernandez de Bolaños, 1979; Horowicz and Schneider, 1981a; Horowicz and Schneider, 1981b) and used as a reporter of the elevated myoplasmic Ca^{2+} resulting from depolarization-induced Ca^{2+} release or, more directly using optical methods and Ca^{2+} indicators introduced in the myoplasm to monitor myoplasmic Ca^{2+} transients (Ashley and Ridgway, 1970; Kovacs et al., 1979; Miledi et al., 1977). The measured change in myoplasmic $[\text{Ca}^{2+}]$ can be used to with a modelling approach to estimate the change in total myoplasmic Ca^{2+} bound to different myoplasmic sites, thereby providing a measure of the total Ca^{2+} released or, from its time derivative, the rate of Ca^{2+} release (Baylor et al., 1983; Melzer et al., 1984; Melzer et al., 1987). More recently developed low affinity Ca^{2+} indicators (Kabbara and Allen, 2001; Launikonis et al., 2005; Ziman et al., 2010), SR-targetable Ca^{2+} indicators (Brini et al., 1997; Canato et al., 2010; Sztretye et al., 2011) and advanced imaging techniques (Launikonis et al., 2005) have allowed the measurement of changes in intra luminal SR $[\text{Ca}^{2+}]$ during various types of fibre activity. In addition, optical techniques and membrane potential-sensitive dyes can be used to monitor the time course of the action potential radial propagation in the TT system (DiFranco et al., 2005; Heiny and Vergara, 1982).

6. Voltage clamp configurations

A key issue in voltage clamping of skeletal muscle fibres is attainment of a spatially uniform membrane voltage. In contrast to the squid giant axon preparation where internal axial wire electrodes were introduced to achieve spatial voltage uniformity (Hodgkin et al., 1952), axial wire electrodes are not appropriate to achieve spatial voltage clamp uniformity in muscle fibres due to the smaller fibre diameter and complicated internal membrane geometry of muscle fibres, so alternative approaches had to be developed. Pioneering work

in muscles isolated from amphibians and crustaceans and subsequent work in mammalian muscle fibre preparations used microelectrode techniques or high resistance gap methods to control the voltage across the fibre.

6.1 Voltage clamp with microelectrodes technique

One approach to achieving space clamp control of membrane voltage in skeletal muscle fibres was to use three microelectrodes near the end of the fibre (Fig. 2A), one microelectrode (most distant from the fibre end) to apply current and two to measure voltage (Adrian et al., 1970). A prerequisite for the use of this arrangement is that fibre movement must be completely eliminated in order to avoid fibre damage at the sites of microelectrode impalement, which was achieved using bathing solutions made highly hypertonic by sucrose addition (Adrian et al., 1970). In general, a voltage change applied near the end of the fibre will cause current to flow across the membrane not only in the short “terminated” segment of fibre having relatively uniform voltage near the fibre end, but also in the longer, essentially infinite cable, length of fibre extending away from the terminated segment and having non-uniform voltage. However, by introducing two voltage recording microelectrodes between the current passing microelectrode and the end of the fibre, the current in the region of relatively uniform voltage near the end of the fibre can be separately monitored as the voltage drop across the internal resistance between the two voltage recording microelectrodes (Adrian et al., 1970; Schneider and Chandler, 1973). For most situations, the current recorded in this way provides an accurate measure of the current crossing the fibre membranes from mid-way between the two voltage recording electrodes to the end of the fibre (Adrian et al., 1970). One major advantage of the 3 microelectrode voltage clamp technique is that it does not require that individual muscle fibres be isolated from the muscle. However, it can be used to record from individual fibres at the end of a dissected whole muscle pinned out in the recording chamber and cleaned of connective tissue at the ends of the muscle fibres (Adrian et al., 1970). With this arrangement, recordings could be made sequentially on multiple fibres within a single muscle by simply removing the microelectrodes from one fibre and reinserting them in another fibre (Chandler et al., 1976). Another advantage is the ability to monitor the actual membrane voltage that has been imposed in the fibre. Major disadvantages include: 1) fibre damage during impaling or fibre movement, 2) slow diffusion of microelectrode solution components into the myoplasm (i.e., channel blockers and Ca^{2+} indicator when used simultaneously with Ca^{2+} imaging techniques) and 3) a more complicated setup (i.e., the requirement of three micromanipulators).

6.1.1 Two microelectrode voltage clamp technique—Alternatively, two microelectrodes can be used to for a two-electrode voltage clamp configuration (Adrian et al., 1969; Heistracher and Hunt, 1969). The use of short muscle fibres, whose length is similar to or shorter than the fibre length constant, improved the longitudinal control of the fibre membrane potential (Caputo et al., 1984; Caputo and Fernandez de Bolaños, 1979; DiFranco et al., 2005; Friedrich et al., 1999; Heistracher and Hunt, 1969; Ursu et al., 2005; Woods et al., 2005). In a variant of this method (Ursu et al., 2005), the electrodes have low resistances (i.e., 2–8 M Ω) (Fig. 2B). This is electrically convenient for both voltage recording and current injection. One of the electrodes is used to continuously record the fibre membrane potential. The other (current-passing) microelectrode, a low resistance suction electrode, can also be used to establish a relatively rapid control of the myoplasmic ionic environment and to deliver Ca^{2+} dyes and Ca^{2+} buffers into the fibre. One major advantage of the two microelectrode voltage clamp technique is that clamp settling times under 0.5 ms are typically possible (DiFranco et al., 2005; Woods et al., 2005), allowing the appropriate recording of membrane currents with fast kinetics (i.e., voltage dependent sodium currents) (DiFranco and Vergara, 2011; Fu et al., 2011). The high current-passing

capacity of the two electrode voltage clamp technique offers the ability to clamp large currents that are impossible to control with single electrode/patch techniques (DiFranco et al., 2011; DiFranco and Vergara, 2011; Fu et al., 2011). However, when working with long fibres (i.e., amphibian semitendinosus or mammalian EDL or soleus fibres) or if the currents under evaluation are extremely large (i.e., in the μA range) the voltage errors can be significant. Another practical problem with the two microelectrode voltage clamp method is capacitive coupling between the electrodes. This potentially causes oscillations that limit the voltage-clamp gain. In most cases, shielding and grounding techniques are required to minimize the coupling. As for the case of whole-cell voltage clamp (see section 6.3), there are commercially available two microelectrode amplifiers as well as user-friendly acquisition software. These amplifiers have circuitries capable of capacitance neutralization of each electrode and bath error potential compensation.

The two electrode voltage clamp technique has been used extensively by several leading groups in the field of skeletal muscle E-C coupling (Andronache et al., 2009; DiFranco and Vergara, 2011; Fu et al., 2011). In summary, the main advantages of the two electrode voltage clamp technique are: 1) high current-passing capacity, 2) fast clamp settling time and the resulting high time resolution, and 3) low noise. The major drawback to the two-electrode voltage clamp is that two microelectrodes must be impaled into the fibre. This requires a more complicated and expensive set-up.

6.2 Voltage clamp with high resistance 'gap' techniques

6.2.1 General considerations regarding cut fibres and gaps—The high resistance gap technique was introduced in pioneering work on skeletal muscle membrane currents (Hagiwara et al., 1969; Hille and Campbell, 1976; Ildefonse and Rougier, 1972; Keynes et al., 1973) and muscle fibre E-C coupling (Kovacs et al., 1979; Kovacs and Schneider, 1978). In this method, a small active region of the fibre is isolated from the rest of the fibre using high resistance gaps. The gaps separate the external solution bathing the active region from the rest of the cell and can be created using air spaces, sucrose solutions or Vaseline. The isolated fibre cut at one or both ends can be studied with a single or multiple gap arrangement that isolates the “internal” solution exposed to cut end(s) from the “external” solution applied to the isolated intact segment of the fibre (Kovacs et al., 1979; Kovacs and Schneider, 1978). The cut end(s) provide a low resistance path to the fibre interior and allows lower noise recordings of membrane currents than those obtained with higher electrical resistance microelectrodes. The cut fibre also allows modification of the internal medium, including the introduction of Ca^{2+} indicator and Ca^{2+} buffers, by modification of the cut end solutions (Kovacs et al., 1979). The segment of the fibre containing the Ca^{2+} -indicator and bathed in the “external” solution can be studied with optical methods. Cut fibres maintain normal function to the extent that the internal solution applied to cut ends mimics the constituents of the myoplasm. Muscle fibres cut after depolarization in relaxing solution and then repolarized after formation of the gap(s) maintain fast electrical propagation of the depolarization signal radially into the fibre via the TT system during membrane depolarizations.

6.2.2 The double gap configuration—For the double gap system (Fig. 2C), voltage is recorded across one gap and the required current for voltage control is applied from the other gap. The total recorded current must be corrected for current leakage via the external resistance across the current passing gap. The voltage recorded across the other (voltage recording) gap corresponds to the voltage across the fibre membranes in the middle pool times the ratio of external resistance to internal plus external resistance ($r_e/(r_e + r_i)$) across the gap, where r_e ⁶ and r_i ⁷ respectively represent the external and internal resistance per unit length across the gap.

6.2.3 The single gap configuration—For the single gap system (Fig. 2D), which provides the possibility of vigorous fibre movement (Horowicz and Schneider, 1981b) or fibre force measurements (Csernoch et al., 1987; Kovacs et al., 1987) of voltage clamped fibres, voltage clamping is then accomplished by first introducing a microelectrode to monitor the voltage in the active region. A compensating circuit is then adjusted to correct for the current flow across the internal and external resistances between the 2 pools of the single gap. Once the compensating circuit is set, the electrode can be withdrawn, and the output of the compensated voltage recording system can be used to voltage clamp the fibre that can then contract vigorously under voltage clamp control (Horowicz and Schneider, 1981b; Kovacs and Schneider, 1978). The compensation settings remain constant providing the values of r_e and r_i do not change. In both the single and double gap systems the current is monitored as total current flow to the active pool, and requires correction for current flow between external solutions across the gap resistance outside the fibre.

Two major advantages of gap methods are the capability of monitoring membrane currents in voltage clamped fibres simultaneously with contractile activity (in the single gap) (Horowicz and Schneider, 1981b; Kovacs and Schneider, 1978) or with Ca^{2+} optical signals (in the double gap) (Kovacs et al., 1979). Some of the disadvantages related to this method are: 1) the current flows across the membrane within the gap contributes to and complicates the total current recorded, 2) cut fibres appear to exhibit an accelerated run-down of Ca^{2+} signals (Wang et al., 1999), presumably due to an accelerated washout of normal constituents, and 3) there is a lack of commercially available amplifiers.

6.3 Whole-cell patch-clamp techniques

Patch clamp recording techniques were developed in the late 1970's (Neher and Sakmann, 1976) and since then has been widely used for investigation of cellular excitability manifested as intramembrane non-linear capacitive currents, transmembrane ionic current and/or generation of action potentials. When operating in the voltage clamp mode, this technique allows the measurement of ionic currents arising either from single channels or through the whole assembly of ion channels expressed in the membrane of both excitable and non-excitable cells (Hamill et al., 1981). One of its variants, the 'whole-cell' configuration, is perhaps the most common configuration used for the measurement of membrane currents. This method has allowed a detailed mechanistic analysis of the gating (i.e., opening and closing) of multiple types of ion channels as well as ion channel modulation by intracellular signalling pathways and pharmacological agents (Cahalan and Neher, 1992). The combination of the patch clamp technique with imaging techniques has expanded our ability to investigate interconnected process such as changes in membrane potential and its coupling to the generation of Ca^{2+} signals in excitable and non-excitable cells (Almers and Neher, 1985; Cahalan and Neher, 1992; Hernandez-Cruz et al., 1990; Neher and Almers, 1986; Thayer et al., 1988; Zhou and Neher, 1993). The patch clamp technology has changed voltage clamping from a complicated technique limited to relatively few laboratories and to a small number of cellular models to a widely applicable and more accessible technique used in virtually every cell type. Another contributing factor to the explosion of the patch clamp studies has been the increase in the availability of high-quality commercial patch clamp amplifiers and user-friendly acquisition and analysis software. However, the successful application of the whole-cell voltage clamp technique requires attainment of spatially uniform voltage, which is difficult to achieve with a long muscle fibre. For this reason the whole-cell clamp was applied early on to the study of the relatively short muscle cells such as embryonic and neonatal skeletal muscle cells (Beam and

⁶external resistance (R_e)

⁷internal resistance (R_i)

Knudson, 1988; Beam et al., 1986; Cognard et al., 1986; Weiss and Horn, 1986), and cardiac myocytes (Bean and Rios, 1989; Cannell et al., 1987; Mitra and Morad, 1985). More recently, the whole-cell clamp technique has been implemented in adult skeletal muscle fibres by using short length muscle fibres (Prosser et al., 2009b; Royer et al., 2008; Wang et al., 1999).

6.4 Membrane current normalized to fibre capacitance

In all voltage clamp configurations, a general consideration is that in order to compare membrane currents recorded in different muscle fibres, it is important to refer the recorded current to the area of fibre membrane actually under study with the recording configuration applied to each fibre. One option would be to determine the membrane area of each fibre under study using microscopic imaging procedures, and calculation of the surface membrane and TT area within the fibre. A very practical alternative approach, which utilizes the same voltage clamp and current recording procedures as the recording of the membrane currents, is to monitor the total linear capacitance of each fibre area under study using pulses covering a relatively hyperpolarized voltage range where there are essentially no voltage dependent processes occurring. The integral of this transient capacitive current is then proportional to the linear capacitance of the fibre or fibre region under study (see section 7.2, below). Other voltage clamp recorded currents in the same fibre can then be normalized to this fibre linear capacitance.

7. Whole cell patch clamp recording in adult skeletal muscle fibres

In the late 1990's the application of the patch clamp technique to short skeletal muscle fibres (Fig. 2E) for the measurement of intramembrane charge movement, Ca^{2+} currents, and Ca^{2+} transients in mouse muscle fibres was pioneered by Delbono's group (Wang et al., 1999), and subsequently used by other groups, including our laboratory (Prosser et al., 2009b; Royer et al., 2008). These studies extended the whole-cell patch-clamp methods to adult skeletal fibres allowing the measurements of voltage dependent membrane currents in combination with estimations of voltage dependent SR Ca^{2+} release. As background, we recommend the eager reader to review the classical description of the patch clamp technique (Hamill et al., 1981; Neher and Sakmann, 1976; Sigworth and Neher, 1980) and the first demonstration of its application to adult skeletal muscle fibres (Wang et al., 1999).

7.1 Recording Solutions

In studies of E-C coupling, the membrane current of primary interest is the gating current of $\text{CaV}1.1$, which generates the E-C coupling intramembrane charge movement. The ionic current carried by $\text{CaV}1.1$ is also of interest, but must generally be studied separately from the charge movement since $\text{CaV}1.1$ ionic current overlaps in time with the later phase of charge movement current records. In order to minimize and/or eliminate other ionic components both the external and internal solutions are formulated to replace major permeable ions (i.e., Na^+ in the external bath solution and K^+ in the internal solution and Cl^- in both external and internal solutions) with non-permeable ionic groups. In addition, the use of more selective channel blockers is also helpful (see (Prosser et al., 2009b; Wang et al., 1999) for composition of the recording solutions).

7.1.1 External solution—The use of tetraethylammonium (TEA)⁸ as a main external cation appears work properly; TEA is a K^+ channel blocker for a diverse group of K^+ channels and is impermeant in Na^+ channels. In order to achieve a more complete blockade of Na^+ and K^+ components addition the external solution must be supplemented with tetrodotoxin⁹

⁸tetraethylammonium (TEA)

(TTX, a Na⁺ channel blocker) and 4-aminopyridine¹⁰ (4AP, a blocking agent for some K⁺ channels). To eliminate Ca²⁺ currents Ca²⁺ channel blockers such as Cd²⁺ and Co²⁺ are used since they may not interfere with E-C coupling charge movement (Adrian et al., 1970; Prosser et al., 2009b; Schneider and Chandler, 1973). We prefer the use of divalent Ca²⁺ blockers instead of trivalent blockers (i.e., La³⁺) or drugs such as dihydropyridines (i.e., nifedipine) since they may respectively profoundly affect surface's potential and channels' gating (Armstrong and Cota, 1990; Bustamante, 1987; Tytgat and Daenens, 1997) and directly the Cav1.1 gating machinery (Lamb, 1986b; Lamb and Walsh, 1987; Rios and Brum, 1987). Chloride current is large in the muscle fibres, and to minimize its contribution we substitute Cl⁻ with the less permeant monovalent anion methanesulfonate (CH₃SO₄) (Hutter and Noble, 1960). This is achieved by combining equimolarly TEA-OH and methanesulfonic acid. While Cl⁻ channel blockers can be also used (i.e., anthracene-9-carboxylic acid, 9-AC¹¹) (Palade and Barchi, 1977) we favour the anion substitution approach. One important prerequisite is to minimize fibre's contraction during seal formation and during acquisition while delivering depolarizing steps. This is achieved by the addition of *N*-benzyl-*p*-toluene sulphonamide¹² (BTS, an inhibitor of myosin II ATPase; 25–50 μM) (Pinniger et al., 2005). Without BTS, seal formation and stable recording are very difficult. Finally use a pH buffer (i.e., 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, HEPES¹³) and use TEA-OH to adjust the final pH to 7.4. When measuring Cav1.1 Ca²⁺ currents we use Ca²⁺ as charge carrier and skip the use Cd²⁺ and Co²⁺.

7.1.2 Internal Solution—As mentioned above, one major advantage of the whole-cell configuration of the patch clamp technique is the possibility of manipulating the fibre's internal environment. For the measurement and isolation of non-linear capacitive currents or Ca²⁺ currents, K⁺, the predominant physiological internal cation, is substituted by monovalent cations impermeable to most K⁺ channels. Cesium, TEA, and *N*-methyl-D-glucamine (NMDG)¹⁴ work appropriately. However, due to their lower mobilities, NMDG and TEA produce series resistances larger than those obtained with equimolar Cs⁺-based solutions (Bean, 1992). One major disadvantage of Cs⁺-based solutions, related to the measurement of non-linear capacitive currents, is that Cs⁺ is not completely impermeant in some K⁺ channels and is also permeable through Ca²⁺ channels. These properties may generate outward Cs⁺ currents carried by both K⁺ and Ca²⁺ channels at extreme positive potentials (Bean, 1992; Horowicz and Schneider, 1981b). Nevertheless, compared with large non-permeable inorganic (NMDG and TEA) or organic (aspartate and glutamate) cations, a large improvement in sealing and durability is obtained with Cs⁺-based solutions (Bean, 1992; Prosser et al., 2009b).

Non-linear capacitive currents and Ca²⁺ currents are more stable if the myoplasmic free Ca²⁺ levels are buffered at low levels. We generally use 10–20 mM ethylene glycol-bis(aminoethylether) *N*, *N*, *N*, *N*'-tetraacetic acid (EGTA)¹⁵ in the internal solution with either no Ca²⁺ added or with Ca²⁺ and Mg²⁺ added to maintain free Ca²⁺ and Mg²⁺ levels close to resting values. The amplitude of the Ca²⁺ currents tends to decrease (run-down) after breakthrough into the whole-cell configuration. The extent and the time course of the rundown are minimized and delayed, respectively, when EGTA and ATP are included in the internal solution. An ATP-regenerative system of creatine phosphokinase and creatine phosphate have been shown to slow rundown even more effectively than ATP and Ca²⁺

⁹tetrodotoxin (TTX)

¹⁰4-aminopyridine (4-AP)

¹¹anthracene-9-carboxylic acid (9-AC)

¹²*N*-benzyl-*p*-toluene sulphonamide (BTS)

¹³4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)

¹⁴*N*-methyl-D-glucamine (NMDG)

¹⁵ethylene glycol-bis(aminoethylether) *N*, *N*, *N*, *N*'-tetraacetic acid (EGTA)

buffering. The rundown process is also effectively reduced by the inclusion of proteases inhibitors (i.e., leupeptin) (Bean, 1992; Prosser et al., 2009a; Prosser et al., 2009b).

7.2 Measuring intramembrane charge movement and Ca^{2+} currents

Movement of the Cav1.1 TT voltage sensors is monitored as intramembrane non-linear capacitive current. The charge movement is estimated from these measurements. Intramembrane non-linear capacitive currents are detected with appropriate procedures for eliminating the larger ionic currents (above) and for measuring and subtracting the linear capacitive currents flowing through the surface and TT membranes (Rios and Pizarro, 1991; Schneider and Chandler, 1973). After the ionic currents have been eliminated, the recording current will include some residual ionic leakage current, as well as linear capacitive current and non-linear capacitive currents. The linear capacitive current is separated from the total current by exploiting one property of non-linear capacitive currents, its saturation. This is an expected property of charge or dipoles that are confined to the membrane. In contrast, the charging of the membrane is not expected to saturate. Therefore if pulses of equal amplitude but opposite polarity ($P+$ and $P-$) are applied at a voltage region where the properties are mostly linear (i.e., -120 mV) it is expected that such pulses will generate linear capacitive current responses of equal amplitude and time course. The algebraic addition of such currents will produce a zero sum response. However, if the positive pulse is applied at a range where the membrane behaves non-linearly, and the negative pulse is still applied at a voltage where the membrane current is linear, the addition of these current responses will completely eliminate the linear current components and isolated the asymmetric current (i.e., non-linear capacitive current, as well as any remaining non-linear ionic current. This method is known as the $\pm P$ procedure. One problem related to this subtraction protocol is that when a large positive pulse is applied, the corresponding negative pulse will reach extremely negative values, a situation that is not well tolerated by cells frequently followed by subsequent membrane damage. The P/n method (Armstrong and Bezanilla, 1974) solves this problem by using small pulses, multiples of the test pulse amplitude. Figure 3 shows an example with subtraction pulses P/4 going in the positive direction. The P/4 applies four pulses, each $\frac{1}{4}$ the amplitude of the test pulse P (Fig. 3A). The current generated by each $\frac{1}{4}$ subtraction pulse is added and then this linear subtraction template (Fig. 3B, red trace) is subtracted from the total current generated by the test pulse (P) (Fig. 3B, black trace) to obtain the asymmetric current (i.e., non-linear capacitive current plus residual ionic components; Fig. 3B, blue trace and Fig. 3C, upper trace). In most cases, the asymmetric current contains ionic components not completely removed by P/4 protocol (Fig. 3C, arrow in top trace shows the steady-state amplitude of this current). The non-linear capacitive current (Fig. 3C, lower trace) can be isolated by subtracting from the asymmetric current a sloping baseline (Fig. 3C) (Chandler et al., 1976). The use of this method is based on the assumption that the residual ionic component is linear and that the slight variation in ionic current during the pulse was linear with time (Chandler et al., 1976). To attain reliable measurements of non-linear capacitive currents is important to set the voltage used to obtain linear current subtraction templates at values where little or no non-linear charge movement is generated, typically to a value more negative than the holding potential.

To evaluate the voltage dependence of charge movement activation, a series of depolarizing steps of increasing amplitude is applied from the holding potential, a membrane potential that is maintained by a patch clamp amplifier. Application of depolarizing pulses leads to transition of sensors into an active state and to measurable current. Typical non-linear capacitive current traces measured from mouse *Flexor digitorum brevis* (FDB)¹⁶ skeletal muscle fibres are illustrated in Fig. 4A. These currents can be represented as $I_Q(t)$. The total

¹⁶Flexor digitorum brevis (FDB)

non-linear charge moved (Q) during the pulse is obtained by calculating the area under the curve of each trace of non-linear capacitive current from the beginning of the pulse (t_0) until the time (t_b) required to achieve the steady-state level,

$$Q = \int_{t_0}^{t_b} I_Q(t) dt, \quad (\text{Eq. 1})$$

In the absence of contamination with ionic current or charge movement immobilization, the amount of charge that moves during the beginning of the pulse (Q_{ON}) must be equal to the charge that moves after the end of the pulse (Q_{OFF}). The amount of Q that moves during a membrane depolarization increases with pulses of increasing amplitude (Fig. 4B). The voltage dependence of charge movement activation (Q vs. V) can be described using a two-state modified Boltzmann distribution (Fig. 4B),

$$Q(V) = Q_{\max} / (1 + \exp((V_{\text{half}} - V)/K)), \quad (\text{Eq. 2})$$

where $Q_{\max}$ gives the maximum charge movement, V_{half} defines the potential where $Q = 0.5$ of $Q_{\max}$ and K is a measure of the steepness of the Q - V relationship. $Q_{\max} = Nze$, and $K = kT/ze$, where, N is the number of moving charged particles, z is the effective valence (i.e., the valence times fraction of field traversed), e is the elementary charge, k is the Boltzmann's constant and T is absolute temperature.

Skeletal muscle fibres represent a favourable native preparation to study Ca^{2+} channel gating charge movement because a large component of the total non-linear capacitive current arises from the voltage sensors for SR Ca^{2+} release (Rios and Pizarro, 1991). Two observations, from early experiments on frog skeletal muscle fibres, support the conclusion that a majority of the charge movement measured in muscle fibres is in fact related to muscle activation. First, the repriming of contraction after long depolarization can be predicted by the restoration of charge movement (Adrian et al., 1976). Second, the decrease in test pulse duration for detectable contraction due to applying a sub threshold prepulse could be predicted as being equal to the time to move the prepulse charge at the test pulse voltage (Horowicz and Schneider, 1981a). These two observations argue that most of the charge that is measured at least has the kinetic characteristics to be consistent with control of Ca^{2+} release. The implication is that the Q for any other channels and/or carriers in the muscle fibre channels would be unlikely to have these kinetic characteristics. Thus, it seems unlikely that a major fraction of the total measured Q is contributed by other channel gating currents.

The activity of several members of the family of voltage operated Ca^{2+} channels is regulated by complex interaction between voltage, Ca^{2+} and extracellular and intracellular messengers (Catterall, 2000; Catterall, 2011). All these Ca^{2+} channels types are able to respond to membrane depolarization by opening a pore, selective for Ca^{2+} , and other divalent cations, and thus generating an inward current into the cells (Catterall, 2011). During sustained depolarizations, Ca^{2+} channels progressively undergo a transition to a non-conducting, inactivated state. This transition can be triggered either by the membrane potential (voltage-dependent inactivation; VDI)¹⁷ or by Ca^{2+} (Ca^{2+} -dependent inactivation; CDI)¹⁸ (Cens et al., 2006; Eckert and Chad, 1984; Peterson et al., 1999). Ca^{2+} dependent inactivation (CDI) has been documented for the great majority of voltage dependent Ca^{2+} channels (Cav) when expressed in heterologous systems, but whether native Cav1.1 of adult skeletal muscle

¹⁷voltage-dependent inactivation (VDI)

¹⁸ Ca^{2+} -dependent inactivation (CDI)

exhibits this feature remains controversial (Ohrtman et al., 2008; Stroffekova, 2008). After repolarization a 'tail' current develops, which is composed of residual non-cancelled linear capacitive current, non-linear charge movement and Ca^{2+} current due to the increase in driving force, followed by Ca^{2+} channel deactivation. To evaluate the voltage dependence of current activation a series of depolarizing steps with increasing amplitude is applied from suitable holding potential, which is maintained by a patch clamp amplifier. The holding potential is set to a value negative enough to reduce the open probability and keep the channels in their closed state(s). For L-type (Cav1.1) calcium channels this value is -80 mV. Application of depolarizing pulses leads to transition of channels into an open state and to measurable inward current. Figure 5A shows exemplar time courses of Ca^{2+} currents from FDB fibres in response to 150 ms depolarizing steps to -40 mV to $+20$ mV as illustrated above the current traces. Current onset was slow and maximum inward current peaked at approximately 80 ms with a pulse to 0 mV. As the reversal potential for Ca^{2+} ions is positive, voltage dependence of current amplitudes (I–V curve) is U-shaped (Fig. 5B). Such voltage dependence can be fitted by Boltzmann-Ohm function, described by the following equation (Nakai et al. 1996):

$$I = G_{\max}(V - V_{\text{rev}}) / [1 + \exp(-(V - V_{\text{half}})/K)], \quad (\text{Eq. 3})$$

where $G_{\max}$ is the maximum conductance, V is the membrane potential, V_{rev} is the reversal potential, V_{half} is the half-activation potential, and K is a measure of the steepness. As demonstrated in Figure 5B, channel I–V relationship close to the reversal potential deviates from the Boltzmann-Ohm equation. Note that theoretical reversal potential for Ca^{2+} is about $+150$ mV while the observed reversal potential is about $+40$ mV. This is caused by loss of ion selectivity in the outward direction in the presence of Cs^+ in the pipette solution. The observed outward current is carried mostly by Cs^+ ions (Bean, 1992). Further details of how to evaluate Ca^{2+} channel activation, deactivation, and inactivation using macroscopic Ca^{2+} currents measurements have been previously described in original papers and reviews (Almers et al., 1981; Delbono and Stefani, 1993a; Jones, 1998; Sanchez and Stefani, 1978).

7.3 Measurements of Ca^{2+} transients

Myoplasmic Ca^{2+} transients generated by Ca^{2+} release from the SR terminal cisternae in response to membrane depolarizations, and the initiating Cav1.1 voltage sensor activation can be measured simultaneously using combined electrophysiological and imaging techniques. Luminescent photoproteins (aequorin) and Ca^{2+} indicator dyes (e.g., arsenazo III; antipyrilazo III, fura-2, fluo-4, etc) have been applied to monitor the changes in myoplasmic $[\text{Ca}^{2+}]$. Advantages and disadvantages of the different Ca^{2+} indicators (speed of reaction, calibration, equilibrium conditions, secondary effects) and main results have been described in recent reviews (Baylor and Hollingworth, 2000; Baylor and Hollingworth, 2011).

The dye can be introduced via the patch pipette after the establishment of whole-cell configuration (Wang et al., 1999). Because of the relative large size of muscle fibre and the relatively low mobilities of some the components the internal solution (i.e., EGTA, Ca^{2+} indicators) a prolonged dialysis (20–60 minutes) time is necessary to achieve a more complete diffusion of the Ca^{2+} indicators and other constituents of the internal solutions (Prosser et al., 2009a; Royer et al., 2008). The Ca^{2+} signals monitored using Ca^{2+} dyes can be detected using different light detecting devices (such as photodiodes, photomultiplier tubes) as well as conventional or confocal microscopy techniques.

The measured $[\text{Ca}^{2+}]$ can be used with a modelling approach to calculate the change in total myoplasmic calcium (CaT) bound to various myoplasmic sites (i.e., troponin C, which controls actin-myosin interactions, and parvalbumin a Ca^{2+} binding proteins in the

myoplasm) and its final re-uptake by the SR. The rate of SR Ca^{2+} release is calculated as $d\text{Ca}/dt$ (Baylor et al., 1983; Melzer et al., 1984; Melzer et al., 1987). Several groups have followed an approach in which unknowns in the removal model are minimized by imposing a large concentration of EGTA via the patch pipette (Gonzalez and Rios, 1993; Prosser et al., 2009a; Ursu et al., 2005; Woods et al., 2005). When $[\text{EGTA}]$ is sufficiently high, only four forms of Ca^{2+} must be considered: free, bound to the monitoring dye, bound to EGTA (Ca-EGTA) and sequestered in the SR. In this condition, EGTA is the major binding site for released Ca^{2+} . The rate of SR Ca^{2+} release in the fibre is approximated from $d[\text{Ca-EGTA}]/dt$. It is believed that EGTA at high concentrations is sufficient to compete with the endogenous troponin C Ca^{2+} binding sites, but sufficiently slowly reacting so as not to buffer $[\text{Ca}^{2+}]$ in the immediate vicinity of the $\text{CaV}1.1$ and RyR Ca^{2+} release channels (Pape et al., 1995). Thus, local Ca^{2+} regulation of these channels may remain despite the binding of the majority of released or entering Ca^{2+} by the internally applied EGTA. Figure 6 illustrates the protocol used for monitoring Ca^{2+} transients in these studies. FDB muscle fibres were voltage clamped at a holding potential of -80 mV. In these experiments, $100 \mu\text{M}$ fluo-4 was included in the patch pipette solution, and was allowed to diffuse into the fibre for 20 min following seal formation. Fig. 6C shows fluo-4 fluorescence Ca^{2+} transients using series of 80 ms step depolarizations increasing in amplitude by 20 mV increments, with a 30 s interval between pulse applications. Confocal line scan ($x-t$) images were acquired at $100 \mu\text{s}/\text{line}$ synchronized to start before each of the pulses during step depolarizations (Fig. 6B). Using the voltage protocol illustrated in Fig. 6C, a detectable fluo-4 fluorescence change was typically first detected in most fibres with pulses to above -40 mV, and saturated with pulses to around $+20$ mV. Fluo-4 records (Fig. 6C) were used to calculate the free cytosolic Ca^{2+} concentration (free $[\text{Ca}^{2+}]$) (Fig. 6D; see (Klein et al., 1988; Prosser et al., 2009a) for more details on the equations used for these calculations). The estimation of the SR Ca^{2+} release flux in each fibre was then calculated from its free $[\text{Ca}^{2+}]$ waveform (Fig. 6E; see (Prosser et al., 2009a; Royer et al., 2008; Shirokova et al., 1996) for more details on the equations used for these calculations). Each of the SR Ca^{2+} release waveforms for the larger depolarizations is characterized by a pronounced early peak that occurs within 10 ms of the application of these larger pulse depolarizations (Fig. 6E). This is then followed by an initial rapid decline and a much slower decline with time in a profile that is believed to reflect an early partial inactivation of the $\text{RyR}1$ and the later effect of a time dependent decline of the SR Ca^{2+} content, respectively (Schneider and Simon, 1988; Schneider et al., 1987). The amplitude of peak SR Ca^{2+} release flux during a membrane depolarization increases with pulses of increasing amplitude. The voltage dependence of peak SR Ca^{2+} release (R vs. V) can be described using a two-state modified Boltzmann distribution,

$$R(V) = R_{\max} / (1 + \exp((V_{\text{half}} - V)/K)), \quad (\text{Eq. 4})$$

where $R_{\max}$ gives the maximum peak SR Ca^{2+} release flux, V_{half} defines the potential where $R = 0.5$ of $R_{\max}$ and K is a measure of the steepness of the R - V relationship.

Whole-cell voltage clamped fibres also exhibit run-down of the Ca^{2+} signals. However the extent of the run-down is less pronounced than that observed using high resistance gap techniques (Wang et al., 1999).

7.4 Series resistance errors

In the whole-cell configuration, a patch pipette is electrically connected in series with the interior of the cell, a situation similar to that of the microelectrodes technique except that the “sharp” microelectrode tip is very small (sub micron internal diameter), but is filled with 3M KCl to minimize its resistance. The patch pipette tip is larger, but is filled with the relatively

high resistivity internal solution and therefore also has considerable electrical resistance. However, in contrast to microelectrode techniques where separate electrodes monitor voltage and pass current, patch clamp amplifiers use a single electrode to record the voltage and to inject current simultaneously, as also done with the single gap configuration as described above. As a result of this, a practical limitation of the patch clamp technique is that the voltage command is given across the combination of the patch electrode and the cell in series (Armstrong and Gilly, 1992; Jones, 1990). For skeletal muscle fibres, with relatively large size and large currents, the resistance of the fibre may be comparable to that of the pipette, causing an appreciable fraction of the commanded pulse to drop across the pipette. This limitation is referred as series resistance errors (Armstrong and Gilly, 1992). Series resistance errors affect both the speed and the steady-state of the voltage clamp conditions. The effects of the series resistance errors on the voltage clamp accuracy will be that the membrane potential will reach the value of the command pulse slowly and unstably. The membrane potential will reach the imposed voltage exponentially, with a time constant equal the product of series resistance and the cell capacitance. The steady-state error is the result of the series resistance and the amplitude of the ionic current. Therefore, the larger the current that is being measured the larger the error (Armstrong and Gilly, 1992).

A useful method to measure the series resistance is to apply a voltage step at a value where the electrical properties of the membrane behave mostly linearly (i.e., a negative step in the region of -120 mV). The expected current response will be a capacitive current with a resistive component. The current response is measured to estimate the time constant (τ) of the capacitive transient, which is given by the product of membrane capacity (C_m)¹⁹ and series resistance (R_s)²⁰ (Armstrong and Gilly, 1992).

$$\tau = C_m R_s, \quad (\text{Eq. 5})$$

Here, C_m includes the capacitance of the sarcolemma and the internal membranes. Since the amplitude of the test pulse is known (V), C_m can be estimated by integrating the capacitive current transient to measure the charge transferred (Q) using,

$$C_m = Q/V, \quad (\text{Eq. 6})$$

Then, R_s can be estimated by dividing the measured τ by C_m .

$$R_s = \tau/C_m, \quad (\text{Eq. 7})$$

Most commercial patch amplifiers have readouts of C_m and R_s , as well as analog circuitries to cancel C_m and to compensate R_s ; however these readouts usually indicate the values of C_m and R_s that has been cancelled and compensated and not the true C_m and R_s , respectively (Jones, 1990). The measured R_s is invariably higher than the resistance of the pipette (R_p). Typically, R_s values are at least twice R_p . This is due, at least in part, to the partial occlusion of the electrode by cellular components. R_s can be compensated, but only partially. Once that R_s value is known, the R_s error can be calculated. This R_s error value is electronically computed by analog circuitry and fed back to the command pulse to compensate for R_s error. The result of this step is to shape the voltage command applied to the cell in an attempt to make the membrane potential change more rapid and accurate. Unfortunately, this feedback is positive, and results in instability and oscillation if overdone (Armstrong and

¹⁹membrane capacity (C_m)

²⁰series resistance (R_s)

Gilly, 1992; Jones, 1990). In general, for measurements using large cells such as muscle fibres, low values of R_s can be obtained by using low resistance electrodes (0.9–1.2 M Ω) and R_s can be compensated up to 60–80%.

The effects of R_s compensation are illustrated in Fig 7. Macroscopic calcium currents were elicited using 80 ms voltage steps to –20, 0 and +20 mV from a holding potential of 80 mV without (Fig. 7A) and with 60 % R_s compensation (Fig. 7B). At first glance, the qualitative properties of both recordings look appropriate. For example, an initial capacitive signal coincides with voltage step in both cases and is followed by the inward calcium current, then during the end of the pulse, the current show a tail current and eventually returns to resting conditions. However, a closer examination of the records reveals two major differences; Ca^{2+} current records in the R_s compensated condition appears larger and its kinetics of both activation (Fig. 7B) and deactivation are faster (Fig. 7C, black trace) when compared with the R_s uncompensated counterpart (Fig. 7A and Fig. 7C, gray trace). Therefore, the effects of lack of R_s compensation are to artifactually underestimate both current amplitude and kinetics (Armstrong and Gilly, 1992; Jones, 1990).

7.5 Spatial uniformity

Another fundamental limitation of the voltage clamp technique, that applies to non-spherical cellular models, such as axons, muscle fibres, and neurons with elaborated projections, regardless of the method used, is that the membrane potential is not completely controlled in parts of the cell electrically far from the electrode(s). This limitation arises from the ‘cable properties’ of such non-isopotential electrical systems (Armstrong and Gilly, 1992; Jack et al., 1975). Let us suppose that we deal with a cable-like cell (i.e., an axon or a muscle fibre) that is uniform, possess only passive electric elements (i.e., no voltage-operated channels) and is infinitely long. An electrode is used to apply a step-like depolarization, current injection will cause the membrane potential to change at the site of where the electrode is located. However, if a second electrode is used to measure the voltage at different points away from the control site, the steady-state voltage will decay as one place the electrode away from the control site. The steady-state solution to the linear cable equation (Jack et al., 1975) is:

$$V=(r_i I_0 \lambda / 2) \exp (-x / \lambda), \quad (\text{Eq. 8})$$

where λ , conventionally is referred to as the length or space constant, is $(r_m/r_i)^{1/2}$, where r_m^{21} and r_i , represent membrane resistance times unit fibres length and internal resistance per unit length, respectively, and I_0 is the applied current. The decay in voltage eventually reaches resting levels falling to 1/e of its initial value in one space constant.

An isolated skeletal muscle fibre in principle can be represented as a ‘passive’ truncated cable of finite length with ends sealed by surface membrane. The equations and solutions to describe the voltage drop across the length of a truncated cable are mathematically more complicated (Armstrong and Gilly, 1992; Jack et al., 1975). In this case, the voltage will drop from the point of control, but with a minor deviation in voltage at the ends (Armstrong and Gilly, 1992). If the length of the fibre is shorter than its λ , the change of membrane potential at the ends of the fibre might be negligible. However, muscle fibres are more complex than a passive truncated cable. They have elaborated membrane infoldings, the TT system, and non-linear ionic components that become active with membrane depolarization. The activation of these components may dramatically reduce the half distance, causing space clamp errors.

²¹ membrane resistance (R_m)

7.5.1 TT system and space clamp errors—The contribution of the TT system to space clamp errors deserves a special consideration. In skeletal muscle the TT system is a specialized membrane network with a complex geometry suited to play a critical role in the E-C coupling process. In one hand, studies of the microarchitecture of mammalian fibres indicate the TT system is distributed uniformly through the fibre cross-section and in a double row of T-tubules per sarcomere (DiFranco et al., 2005; Dulhunty, 1989; Franzini-Armstrong et al., 1998; Revel, 1962). The triads, the basic structural assemblies, consist of one T-tubule facing two SR terminal cisternae (TC), located at both sides of the Z-line at the I-A junction (Fig 1A). On the other hand, biophysical evidence indicates that the TT system has linear and non-linear electrical properties in direct continuity with the extracellular space at the fibre surface. Further evidence obtained in amphibian and mammalian fibres has demonstrated that the TT system is indeed an active network with a voltage dependent sodium conductance critical for the radial propagation of surface's action potential towards the fibre centre, subsequent voltage sensor activation causing Ca^{2+} release and muscle contraction (Adrian et al., 1969; Bezanilla et al., 1972; González-Serratos, 1971; Heiny and Vergara, 1982). A prerequisite to study the voltage dependence of the charge movement, Ca^{2+} current and Ca^{2+} release is that the experiments must be conducted in voltage-clamped fibres whose surface sarcolemma and TT systems are rendered passive by blocking or minimizing critical conductances (i.e., Na^+ , K^+ and Cl^-). Under these conditions the TT system can be represented electrically as a radial cable with multiple elements and a large distributed capacitance separated from membrane surface by an access resistance (Adrian and Peachey, 1973) and a distributed T-tubule lumen resistivity (Adrian et al., 1969; Falk and Fatt, 1964; Schneider, 1970). All these characteristics might impose an experimental limitation to the biophysical characterization of the signals originated or controlled by the TT system. For example, the voltage excursion experienced by the TT system may differ significantly in amplitude and speed from the voltage imposed at the surface sarcolemma. These limitations were initially observed in voltage clamp experiments using high resistance gap techniques conducted simultaneously with optical potentiometric measurements in the TT system using large amphibian muscle fibres (Ashcroft et al., 1985; Heiny and Vergara, 1982; Jong et al., 1997). In response to a step-like depolarization applied at the surface, the average TT system potential reached a steady-state value only after 20–30 ms (Ashcroft et al., 1985; Heiny and Vergara, 1982; Jong et al., 1997). These limitations made charge movements and Ca^{2+} signals obtained with voltage clamp techniques difficult to interpret on the time scale of an action potential (Heiny and Vergara, 1982; Kim and Vergara, 1998b). Based on the method developed by Armstrong and Chow (Armstrong and Chow, 1987), Vergara and collaborators, using high resistance gap techniques in parallel with potentiometric measurements, implemented a supercharging method for the muscle fibres (Kim and Vergara, 1998a; Kim and Vergara, 1998b). In this method, a triexponential decay waveform was summed at the start and end of the voltage clamp depolarization. Using this technique, they were able to demonstrate that supercharging command pulses could be used to establish voltage clamp settling times as fast as 1.5 ms in the TT system of semitendinosus muscle of the frog. The supercharging method also accelerated significantly the charge movement and Ca^{2+} release from the SR (Kim and Vergara, 1998b).

Subsequently, using the two electrode voltage clamp technique in combination with optical potentiometric measurements in the TT system it was demonstrated that, unlike what was found in frog muscle, in mouse FDB muscle fibres the time course of TT system potentiometric signals elicited by action potentials and voltage pulses were virtually undistinguishable from those at the fibre surface membrane (DiFranco et al., 2005; Woods et al., 2005).

In skeletal muscle fibres, surface and radial cable length constant values are around 1 mm and 60 μm , respectively (Adrian et al., 1969; Chandler et al., 1976). In general, to minimize

space clamp errors it is highly recommended use short (300–500 μm in length) and thin fibres (20–30 μm in width). In voltage clamp conditions a method to test for longitudinal voltage uniformity under real experimental conditions is to measure the membrane potential using a second electrode at various points along the fibre (Armstrong and Gilly, 1992).

Alternatively, one can combine membrane potential measurements using a voltage sensitive dye (DiFranco et al., 2005) together with voltage clamp depolarizations. The use of high-speed line scan confocal acquisition technology combined with potentiometric dyes (i.e., di-8-butyl-amino-naphthyl-ethylene-pyridinium-propyl-sulfonate (di-8-ANEPPS)²²) in a voltage clamped fibre subjected to step depolarizations allows the measurements of changes in membrane potential at the TT system. Figure 8 presents di-8-ANEPPS fluorescence measurements at three locations 50, 100 and 150 μm away from the patch pipette (Fig. 8A). Figure 8B illustrates representative line scan (x - t) di-8-ANEPPS fluorescence records elicited by a 80 ms step depolarization to 0 mV. The di-8-ANEPPS signal amplitude is similar at the three different locations where it was measured (Fig. 8C), however the steady-state values are attained with a ~ 1.5 –2 ms delay (Fig. 8D; see figure legend). Figures 8E–G illustrate the TT system depolarization measured at the edges and at the centre of the fibre (Fig. 8E). The rising time for the di-8-ANEPPS signal at the centre of the fibre was slightly slower than at the edges (Fig. 8G; see figure legend). These potentiometric measurements show that a decent clamp uniformity, both longitudinally and radially across the fibre, is achieved under whole-cell voltage clamp when using relatively short fibres.

Another indirect way to evaluate spatial homogeneity of the clamp requires the combination of voltage clamp techniques in parallel with Ca^{2+} imaging techniques. In this method Ca^{2+} transients elicited by membrane depolarizations of constant amplitude are measured at different locations away from the patch pipette and then the time course of the Ca^{2+} signals is compared. Figure 9 illustrates the time course of fluo-4 (expressed as $\Delta F/F_0$) using line-scan (x - t) confocal measurements in a fibre subjected to voltage clamp and sequential 80 ms step depolarizations to 20 mV evaluated near to the patch pipette (Fig. 9D–E, black trace) located around the middle of the fibre (Fig. 9A–B) or about 100 μm away from the pipette (Fig. 9D–E; green and red traces), near to fibre's ends (Fig. 9A–B). Figures 9F–H illustrate the time course of the fluo-4 signals elicited by a step depolarization measured through the fibre section at the edges and at the centre of the fibre (Fig. 9F). The fluo-4 signals are almost identical at the three different radial locations within the fibre (Fig. 9G–H). These fluo-4 measurements show that appropriate Ca^{2+} transient uniformity, both longitudinally and radially across the fibre, is achieved under whole-cell voltage clamp conditions.

7.6 Size matters: *Flexor digitorum brevis* muscle fibres from CD1 vs. C57 mice

Our procedures have been optimized for skeletal muscle fibres isolated from laboratory mice. In the case of adult muscle fibres, one important prerequisite to achieve appropriate voltage clamp conditions is the size of the muscle fibre under study. Fibres enzymatically dissociated from the adult mouse toe muscle FDB provide a good source of short skeletal muscle fibres (Bekoff and Betz, 1977; Bischoff, 1986; Liu et al., 1997; Lupa and Caldwell, 1991). FDB muscle is composed of different fibre populations, with different lengths and expressing three myosin isoforms: I, IIA and IIX. Type I, IIA and IIX fibres constitute 2%, 19% and 21% of all FDB fibres, respectively, and the majority of FDB fibres are hybrids containing more than one myosin isoform (Banas et al., 2011; Raymackers et al., 2000). In our initial experiments aiming to establish the use of the patch clamp in adult muscle fibres we noticed differences in muscle fibre size when comparing predominant short fibres isolated from different mouse strains. Figures 10A and B illustrate this difference when

²²di-8-butyl-amino-naphthyl-ethylene-pyridinium-propyl-sulfonate (di-8-ANEPPS)

comparing typical short FDB fibres isolated from CD1 and C57 strains. Note that fibre geometrical dimensions and corresponding electrical parameters are quite different. Average fibre length, width and linear capacity were $578 \pm 4.9 \mu\text{m}$, $34 \pm 0.4 \mu\text{m}$ and $3.05 \pm 0.46 \text{ nF}$ for CD1 fibres and $398 \pm 2.7 \mu\text{m}$, $29 \pm 0.4 \mu\text{m}$ and $1.57 \pm 0.18 \text{ nF}$ for C57 fibres.

The impact of fibre size on Ca^{2+} current measurements is better appreciated when a side by side comparison is made. Fig. 10 shows representative Ca^{2+} records obtained from FDB fibres isolated from a C57 (Fig. 10C) or CD1 (Fig. 10D) mouse strains. In both cases, the fibres were subjected to voltage clamp via whole-cell patch clamp configuration, using the same internal and external solution designed to isolate Ca^{2+} currents. Fibres were dialyzed for 20 minutes after the breakthrough into whole-cell configuration. Note that the CD1 fibre exhibits an outward ionic component, presumably a K^+ current that has been not completely blocked, due to the larger fibre size and consequent requirement for longer time for diffusional equilibration of the pipette solution with the cytoplasm throughout the fibre interior (Fig. 10E). This current is already active at -60 and becomes more pronounced at -20 mV . In contrast, the outward component appears eliminated at voltages between -60 and -20 mV in the C57 fibre, presumably due to a more effective blockade of K^+ channels in the relatively smaller C57 fibre (Fig. 10D). In addition, peak Ca^{2+} current at 0 mV is larger in the CD1 fibre (-23 nA for CD1 fibre vs. -9.1 nA for C57 fibre) and the currents exhibit some capacitive artefacts at both the beginning and the end of the pulse (Fig. 10E) not seen with the C57 fibre.

In short FDB muscle fibres, recordings of non-linear capacitive currents and Ca^{2+} currents using the whole-cell patch technique indicate that the steady-state accuracy of the clamp is greatly improved. However, the speed of the clamp is slower than that obtained with other techniques such as microelectrodes (Chandler et al., 1976; DiFranco et al., 2005; Sanchez and Stefani, 1978; Schneider and Chandler, 1973; Woods et al., 2005) or high resistance gaps (Delbono, 1992; Hille and Campbell, 1976; Kovacs et al., 1979; Kovacs and Schneider, 1978). Ideally, the voltage change should be instantaneous. In practice, the voltage change using the whole-cell patch clamp techniques is fast with respect of the kinetics of the non-linear capacitive currents and Ca^{2+} currents and the Ca^{2+} transients elicited by depolarizations. Therefore, the patch clamp technique is well suited for the study of charge movement, macroscopic Ca^{2+} currents and Ca^{2+} signals in adult muscle fibres. In contrast, the patch clamp method would not be appropriate to study membrane currents with fast kinetics such as voltage dependent sodium channels, or large current such as some K^+ or Cl^- currents. A more comprehensive treatment of possible errors and limitations related to the whole-cell patch clamp technique can be found in earlier reviews (Armstrong and Gilly, 1992; Jones, 1990).

8. Additional practical concerns related to the voltage clamp methods

One common advantage of voltage clamp technique is that the amplifiers and acquisition and analysis software have become commercially available and easy to operate. For the new user of the voltage clamp techniques, it is often easy to become familiar with the practical aspects of these techniques. However, this had led to one disadvantage; the use of the equipment without careful evaluation of the validity of the acquired data.

Most of the main problems originate during data acquisition and arise from inappropriate leak subtraction procedures, choice of holding potential or stimulation protocols. It is highly recommended to carefully inspect the leak pulses to ascertain that no non-linear current components are present in the subtraction templates (Armstrong and Bezanilla, 1974). Several voltage operated ion channels undergo steady-state and/or use dependent inactivation (Almers et al., 1981; Delbono and Stefani, 1993a; Sanchez and Stefani, 1978).

These processes should be taken into account when developing stimulation protocols to minimize excessive run-down of the signals, and when needed preventive or corrective approaches should be included (Olojo et al., 2011).

Electrode stability is also a concern for any voltage clamp technique. The test electrode and reference electrode in the bath are the interfaces that electrically connect the patch pipette, the cell, and recording bath chamber to the voltage clamp amplifier. The test electrode is a silver wire coated with AgCl. The reference electrode is also a silver wire coated with AgCl or an AgCl pellet. The degradation of the AgCl coat, when large currents are passed, or scratching, due to repetitive use, leads to improper electrode function and inaccurate measurements. It is recommended to regularly monitor electrode stability and when needed rechloride or replace to avoid shifts in the electrode potential.

Finally, differences in osmolarity and pH between the external and internal solutions can dramatically affect the properties of membrane currents. The use of added divalent ions in the bath chamber can modify surface membrane potential, leading to shifts in both activation and inactivation curves of voltage operated channels (Latorre et al., 1992). In addition, complete removal of Ca^{2+} ions from the bath should be avoided. This procedure leads to several effects including loss of Ca^{2+} channel's selectivity (Almers and McCleskey, 1984) and negative shift in the activation curve of voltage operated Na^{+} channels (Campbell and Hille, 1976). When using organic solvents (i.e., dimethylsulfoxide, ethanol) in the external solution keep its concentration $>0.1\%$ and use an agar bridge, to avoid poisoning the reference electrode (Alvarez-Leefmans, 1992).

9. Conclusions

There is no ideal or perfect method for voltage clamping skeletal muscle fibres. Each technique carries its own advantages and disadvantages. Some important factors to take into account are the size of the fibre, and both the amplitude and kinetics of the current under study. Since the speed of the clamp is determined by the product of R_s and C_m , small fibres are preferred, and can be clamped more rapidly and accurately than large fibres. The whole-cell patch clamp and the two electrode voltage clamp techniques are appropriate methods to study E-C coupling in short FDB muscle fibres from the mouse. Low noise non-linear capacitive current and Ca^{2+} current measurements, and depolarization evoked Ca^{2+} transients can be studied with these techniques. High resistance gaps and two microelectrode techniques combined with supercharging voltage commands are highly recommended when working with large fibres (i.e., fibres from amphibian muscle, or long fibres from mammalian muscle). A relatively new technique, the 'silicone' patch clamp technique (Pouvreau et al., 2007), a hybrid of high resistance gaps and patch clamp technique, may be useful to work with long fibres. In this method, a large segment of the fibres is covered with silicone, a high resistive material, aimed to reduce surface area of the fibres. The uncovered region is used to measure membrane currents and Ca^{2+} signals. The two microelectrode and high resistance gaps techniques are preferred when aiming to characterize currents with fast kinetics (i.e., voltage operated Na^{+} current) and/or large amplitude (>10 nA, i.e., voltage operated chloride current).

Voltage-clamp techniques and their combination with Ca^{2+} imaging techniques and molecular biological tools (such as interference RNA, over-expression of proteins and genetically engineered animal models) constitute powerful tools to approach many biophysical and molecular aspects of skeletal E-C coupling. With many remaining unanswered questions about E-C coupling in both physiological and pathophysiological conditions, it is clear that the voltage clamp technique and its combination with cellular and

molecular biological tools will continue to provide a productive approach to explore in further detail the mechanisms that link muscle excitability to the contractile process.

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Figure 1.

Cellular and molecular organization of components of excitation-contraction coupling in a skeletal muscle fibre. (A) Cartoon of a segment of a mammalian skeletal muscle fibre showing the external membrane systems sarcolemma and transverse tubules (TT) and their relationship with the terminal cisternae (TC) of the sarcoplasmic reticulum (SR), the myofibrils and the sarcomere. In adult mammalian skeletal muscle fibres the transverse tubules are located as a pair in each sarcomere at the A-I junction level. (B) Location and relations of the voltage sensors of the T-tubule system, the dihydropyridine receptor (DHPR; also known as Cav1.1) and the Ca^{2+} release channel, the ryanodine receptor (RyR) of the SR. Panel B is a zoomed in version of the rectangle shown in panel A. (C) Subunits and domains of the Cav1.1 channels and their distribution within the TT membrane. The S4 segments of each domain is positively charged and is thought to undergo conformational changes upon depolarizations that are transduced into conformational changes in the RyR that activate the Ca^{2+} conduction pathway via molecular coupling between the DHPR and the RyR. The loop II-III of the alpha 1 subunit is a critical element for the interaction with the RyR. The beta subunit interacts with loop I-II of the alpha1 subunit and modulates both alpha 1 subunit expression and its biophysical properties. The beta subunit appears to interact with the RyR to regulate E-C coupling. Only a section of the RyR 'foot' is depicted. Based on (Beam and Horowicz, 2004; Catterall, 1991; Catterall, 2000; Catterall, 2011; Franzini-Armstrong and Jorgensen, 1994; Melzer et al., 1995; Peachey, 1965; Rios and Pizarro, 1991).

Figure 2. Simplified representation of voltage clamp methods and circuits used in muscle electrophysiology for the study of E-C coupling. (A) Three microelectrode technique. (B) Two microelectrode technique. (C) 'Double' vaseline gap. (D) 'Single' vaseline gap. (E) Whole-cell patch clamp technique. See text in sections 6 and 7 for further details.

Figure 3.

Subtraction of linear components of membrane current using the P/4 protocol and baseline removal. (A) Voltage protocol (top) and corresponding current responses (bottom). Membrane current during a voltage pulse, P , was partially corrected by analog subtraction of linear components using the amplifier capacitance cancelation circuitry. The remaining linear components were digitally subtracted using four pulses (a–d) of 1/4 test pulse P amplitude applied from a -120 mV subholding membrane potential (sHp). (B) Current records in response to an 80 ms step depolarization to 0 mV from a holding potential of -80 mV. Total current (black trace), linear current response template obtained with the P/4 protocol ($a+b+c+d$; red trace) and the isolated asymmetric capacitive current (blue trace) that result from the subtraction of the linear template from the total current. (C) Subtracted current (blue trace) from panel B is shown in a y-axis expanded version after P/4 subtraction (asymmetric current; top trace) and with baseline subtraction (non-linear capacitive current; bottom trace), to remove residual ionic components. In some cases, the P/4 subtracted current contains an ionic component that is not completely removed (arrow in top trace show the steady-state amplitude of this current). Assuming that this ionic component is constant (area under trace) or changing linearly with time, it can be removed by constant or sloping baseline subtraction respectively. The current trace after P/4 and baseline subtraction is the non-linear capacitive current. The dotted lines in traces indicate the zero current reference. See text in section 7.2 for more details.

Figure 4.

Non-linear capacitive currents and intramembrane charge movement in a FDB muscle fibre using the whole-cell configuration of the patch-clamp technique. (A) Representative nonlinear capacitive currents elicited by 80 ms step depolarization of increasing amplitude, to -40 to $+20$ mV using 10 mV increments. Voltage protocol is shown at the top. Non-linear capacitive currents were recorded after the ionic components were blocked or minimized using a mixture of Na^+ , K^+Cl^- and Ca^{2+} channel blockers and ionic substitution manipulations to the recording solutions. (B) Intramembrane charge movement vs. voltage relationship (Q - V). Intramembrane charge movement was calculated as the integral of the current in response to depolarizing pulses (Q_{ON}) normalized to membrane capacitance. The continuous line through the symbols is the fit of Eq. 2 to experimental data.

Figure 5. Voltage dependent Ca^{2+} currents in a FDB muscle fibre using the whole-cell configuration of the patch-clamp technique. (A) Representative Ca^{2+} currents elicited by 80 ms depolarizing pulses to -40 to +20 mV from a holding potential (H_p) -80 mV. (B) Peak Ca^{2+} current vs. voltage relationship ($I_{\text{Ca}}-V$). The continuous line through the symbols is the fit of Eq. 3 to experimental data.

Figure 6.

Fluo-4 transients, Ca²⁺ transients, and Ca²⁺ release flux in a FDB muscle fibre obtained using the whole-cell configuration of the patch-clamp technique. (A) Left, transmitted light image of a segment of an FDB muscle fibre showing the location where the patch pipette was placed. Right, x-y fluorescence image of fibre loaded with 100 μ M fluo-4 (salt) via the patch pipette. The white vertical line indicates the place that was used to obtain high-speed line scan (x-t) confocal measurements. (B) Typical line scan (x-t) confocal image obtained from a fibre loaded with fluo-4 and subjected to voltage clamp step depolarizations. Voltage protocol is shown on top. Note the change in fluoresce elicited by the depolarizing step to -20 mV. White box on image indicates the region of interest (ROI) used to estimate the fluoresce time course. (C) Time course of fluo-4 fluorescence in response to 80 ms step depolarizations to -40 to +20 mV from a holding potential (Hp) of -80 mV. (D) Time course of depolarization-induced changes in myoplasmic free [Ca²⁺] estimated from traces in panel C. (E) Time course of the depolarization-induced Ca²⁺ release flux estimated from traces in panel D, obtained as the time course of the calculated change in Ca²⁺ bound to the 20 mM EGTA applied to the fibre interior via the patch clamp electrode. (F) Peak Ca²⁺ release flux vs. voltage relationship (R-V). The continuous line through the symbols is the fit of Eq. 4 to experimental data.

Figure 7.

Effects of series resistance (R_s) compensation on Ca^{2+} current amplitude and kinetics. Representative Ca^{2+} current records elicited with 300 ms step depolarizations to -20 mV (blue trace), 0 mV (black trace) and +20 mV (red trace) in a fibre where R_s was left uncompensated (A) and from same fibre after 60% R_s compensation (B). (C) Ca^{2+} currents traces in a time expanded scale (dotted box in A and B indicate the zoomed region) at the end of test pulses to 0 mV from panels A and B without R_s compensation (grey trace) and with 60% R_s compensation (black trace). Note the effects of R_s compensation on the Ca^{2+} current amplitude and kinetics. See text section 7.4 for more details.

Figure 8.

Di-8-ANEPPS potentiometric signals elicited by step depolarizations recorded near and away from the patch pipette in a FDB muscle fibre. (A) *Top*, transmitted light image of an FDB muscle fibre showing the location of the patch pipette. *Bottom*, confocal x - y image of the fibre stained with the voltage sensitive dye di-8-ANEPPS (2 μ M; for 1 hr at 37° C). The yellow dashed vertical lines (a – c) indicate the locations that were used to obtain high speed line scan (x - t) confocal measurements. (B) Line scan x - t confocal images obtained sequentially at three different locations (a , near to the patch pipette $x=0$; b and c , 50 and 150 μ m away from the patch pipette, respectively) from a fibre stained with di-8-ANEPPS and subjected to 80 ms step depolarizations to 0 mV using the whole-cell configuration of the patch-clamp technique. Voltage protocol is shown on bottom. (C) Time course of di-8-ANEPPS records from panel B in response to 80 ms step depolarizations to 0 mV (gray trace) evaluated at three different longitudinal locations and measured at regions of interest (ROI) indicated in panel B a – c (dashed white rectangles). (D) Traces from panel C are shown in a time expanded scale. Continuous lines represent least-squares fits to the traces shown, using a function of the form: $y(t)=y_0+A(1-e^{-t/\tau})$. For the signals measured at locations a , b and c , τ , the corresponding time constant values, were 1.4 ms, 1.8 ms and 1.5 ms, respectively. Note that change in fluorescence elicited by the depolarizing step is very similar in the three different locations, indicating good longitudinal space clamp conditions. (E) Line scan image recorded at position Aa (same image as Ba), but now labelled to show ROIs selected at three different radial locations. (F) Time course of di-8-ANEPPS measured at different regions into the fibre. Regions of interest at the edges and centre of fibre are indicated in panel E (red rectangle, ROI-1 at edge; green rectangle, ROI-2 at centre and yellow rectangle, ROI-3 at opposite edge). (G) Traces from panel F are shown in a time

expanded scale. Continuous lines represent least-squares fits to the traces shown, using same function as in panel D. For the signals measured at the edge τ , the time constant, was ~ 1.9 ms; in the centre of the fibre τ was 3.3 ms. The rising phase of di-8-ANEPPS transient obtained from the centre of the fibre showed somewhat slower response than those evaluated at the edge of the fibre.

Figure 9.

Ca^{2+} transients recorded near and away from the patch pipette during a voltage clamp step depolarization of a FDB muscle fibre. (A) Transmitted light image of a FDB muscle fibre showing the location of the patch pipette. (B) Confocal fluorescence x - y image of fibre loaded with 100 μM fluo-4 via the patch pipette. The green dashed vertical lines (a - c) indicate the locations that were used to obtain high-speed line scan (x - t) confocal measurements. (C) x - t confocal images obtained sequentially at three different locations (b and c at the ends of the fibre, about 100 μm away from the patch pipette; a , close to patch pipette) of a fibre loaded with fluo-4 and subjected to voltage clamp steps to -20 mV. Voltage protocol is shown at the bottom. (D) Time course of fluo-4 fluorescence from panel

C in response to 80 ms step depolarizations to 20 mV evaluated at the locations shown in panel B and measured at regions of interest (ROIs) indicated in panel *Ca-c* (dashed white rectangles). (E) Traces from panel D are shown in a time expanded scale. Note that the change in fluoresce elicited by the depolarizing step is very similar in the three different locations, an indication of longitudinal Ca^{2+} transient uniformity. (F) Line scan image recorded at position *Ba* (same image as *Ca*), but now labelled to show ROIs selected at three different radial locations. (G) Time course of fluo-4 signals measured at different radial regions. Regions of interest at the edges and centre of fibre are indicated in panel F (red rectangle, ROI-1 at edge; green rectangle, ROI-2 at centre and yellow rectangle, ROI-3 at opposite edge). (H) Traces from panel G are shown in a time expanded scale. Note that the change in fluoresce elicited by the depolarizing step is very similar in the three different locations within the fibre, an indication of radial Ca^{2+} transient uniformity.

Figure 10.

Comparison of FDB fibres isolated from C57 and CD1 mice. Transmitted light images of typical short FDB fibres isolated from C57 (A) and CD1 (B) mouse. In both the C57 and CD1 strains, the majority of the fibre population is composed of relatively short fibres, with a small fraction composed of relatively long fibres. Note the difference in fibre size. Linear capacitive currents recorded in C57 (C) and CD1 (D) FDB muscle fibres. Records illustrate typical capacitive current records elicited by a 5 mV depolarizing step (to -75 mV) from the holding potential (Hp, -80 mV). The area under the curve was integrated to obtain charge (Q) and estimate total fibre membrane capacitance according to Eq. 6. Capacitance values are shown. Representative Ca^{2+} current records evoked with 80 ms step depolarizations to -60 to $+20$ mV, in increments of 20 mV, from a Hp -80 mV obtained from C57 (E) and CD1 (F) FDB fibres. Note that both capacitive current and Ca^{2+} current are larger in CD1 FDB fibre. Also, note the presence of an outward ionic component elicited by pulses to between -60 and -20 mV in CD1, despite 20 minutes dialysis with a Cs-based internal

solution. In contrast, this outward ionic component is completely absent in C57 FDB fibres. Both C57 and CD1 were subjected to voltage clamp using the whole-cell patch clamp configuration, with exactly the same recording solutions. Records were obtained exactly 20 minutes after the breakthrough into whole-cell configuration. See text section 7.6 for further details.