Role of physiological ClC-1 Cl⁻ ion channel regulation for the excitability and function of working skeletal muscle

Thomas Holm Pedersen,[1] Anders Riisager,[1] Frank Vincenzo de Paoli,[1] Tsung-Yu Chen,[2,3] and Ole Bækgaard Nielsen[1]

[1]Department of Biomedicine, Aarhus University, 8000 Aarhus C, Denmark
[2]Center for Neuroscience and [3]Department of Neurology, University of California, Davis, Davis, CA 95618

Electrical membrane properties of skeletal muscle fibers have been thoroughly studied over the last five to six decades. This has shown that muscle fibers from a wide range of species, including fish, amphibians, reptiles, birds, and mammals, are all characterized by high resting membrane permeability for Cl⁻ ions. Thus, in resting human muscle, ClC-1 Cl⁻ ion channels account for ∼80% of the membrane conductance, and because active Cl⁻ transport is limited in muscle fibers, the equilibrium potential for Cl⁻ lies close to the resting membrane potential. These conditions—high membrane conductance and passive distribution—enable ClC-1 to conduct membrane current that inhibits muscle excitability. This depressing effect of ClC-1 current on muscle excitability has mostly been associated with skeletal muscle hyperexcitability in myotonia congenita, which arises from loss-of-function mutations in the *CLCN1* gene. However, given that ClC-1 must be drastically inhibited (∼80%) before myotonia develops, more recent studies have explored whether acute and more subtle ClC-1 regulation contributes to controlling the excitability of working muscle. Methods were developed to measure ClC-1 function with subsecond temporal resolution in action potential firing muscle fibers. These and other techniques have revealed that ClC-1 function is controlled by multiple cellular signals during muscle activity. Thus, onset of muscle activity triggers ClC-1 inhibition via protein kinase C, intracellular acidosis, and lactate ions. This inhibition is important for preserving excitability of working muscle in the face of activity-induced elevation of extracellular K⁺ and accumulating inactivation of voltage-gated sodium channels. Furthermore, during prolonged activity, a marked ClC-1 activation can develop that compromises muscle excitability. Data from ClC-1 expression systems suggest that this ClC-1 activation may arise from loss of regulation by adenosine nucleotides and/or oxidation. The present review summarizes the current knowledge of the physiological factors that control ClC-1 function in active muscle.

Introduction

The ClC gene family contains structurally related Cl⁻ ion channels and Cl⁻/H⁺ exchangers that are found in a wide range of organisms from bacteria to mammals (Stauber et al., 2012). The ClC-1 Cl⁻ ion channel is the skeletal muscle–specific member of the ClC gene family (Jentsch et al., 1990; Koch et al., 1992; Miller, 2006), and it is responsible for ∼80% of the resting membrane conductance (G_M) in inactive muscle fibers. Because of this high membrane conductance and because Cl⁻ has an equilibrium potential that is close to the resting membrane potential of muscle fibers, ClC-1 dominates the inhibitory membrane current that counteracts action potential excitation in muscle. An inverse relationship between ClC-1 function and muscle excitability is most vividly illustrated by the hyperexcitability of skeletal muscle in myotonia congenita, a muscle disease resulting from loss-of-function mutations in the ClC-1 gene (Koch et al., 1992). Although this role of ClC-1 in myotonia congenita is well established, it has been less clear whether regulation of ClC-1 occurs under different physiological conditions, including muscle activity. Nevertheless, regulation of ClC-1 in active muscle was indicated by observations of expressed ClC-1 being sensitive to a range of cellular signals and metabolic alterations that develop in working muscle, including PKC activation (Rosenbohm et al., 1999), intracellular acidification (Tseng et al., 2007), loss of adenosine nucleotides, and oxidation (Bennetts et al., 2005, 2007; Tseng et al., 2007, 2011). However, after developing the appropriate methodology, it has been shown that the excitability of active muscle is highly dependent on acute regulation of ClC-1 (Pedersen et al., 2009a,b; de Paoli et al., 2013). Here we review this recent work on intrinsic regulation of Cl⁻ channels in healthy muscle during muscle activity. We focus on the role of ClC-1 Cl⁻ channels for skeletal muscle excitability and function from a physiological viewpoint while relating this regulation to prevailing understanding of ClC-1 channel structure.

Correspondence to Thomas Holm Pedersen: thp@biomed.au.dk

Abbreviations used in this paper: CBS, cystathionine-β-synthase; EDL, extensor digitorum longus.

Role of Cl⁻ channel in skeletal muscle: Lessons learned from myotonia congenita

In 1876 the Danish physician Julius Thomsen described how he and several of his family members suffered from spontaneous muscle contractions and delayed muscle relaxation. He suggested that these symptoms reflected an unknown inheritable disease (Thomsen, 1876), and over the next 50–60 yr, it was heavily debated whether Thomsen's disease had a nervous or muscular origin. In 1939, experiments on "fainting" or myotonic goats, which have symptoms that are very similar to those observed in the Thomsen family members, showed that neither denervation nor curare abolished the muscle hyperexcitability (Brown and Harvey, 1939). It was furthermore demonstrated that a local intramuscular infusion of KCl triggered prolonged electrical discharges and substantial contractile activity in myotonic goats, whereas no response was observed in normal goats. Similar activation of myotonic muscle was not observed with intramuscular NaCl infusion. Retrospectively, these findings demonstrated that depolarization induced by local elevation in extracellular K^+ was able to trigger action potentials in muscle fibers of goats with myotonia congenita because they had less inhibitory Cl^- membrane current to stabilize the resting membrane potential. These findings represent a very early demonstration of the important role of the muscle Cl^- channels for muscle excitability. At the time, the observations with denervation, curare, and intramuscular KCl infusion were taken as clear evidence that Thomsen's disease, which was by then also known as myotonia congenita, was indeed a muscle disorder, although its etiology remained unknown.

In 1949, Bernard Katz used extracellular electrodes to show that a large component of G_M in resting amphibian muscle is abolished when Cl^- in the extracellular solution is substituted with nonpermeable anions (Katz, 1949). With the later development of the glass microelectrode for intracellular recordings (Ling and Gerard, 1949), the observations by Katz were confirmed, and quantitative analysis showed that the Cl^- membrane conductance (G_{Cl}) accounts for ∼80% of G_M in resting muscle fibers from amphibian (Hutter and Noble, 1960; Hutter and Warner, 1967c), reptiles (Adams, 1989), birds (Morgan et al., 1975), and mammals (Lipicky et al., 1971; Palade and Barchi, 1977).

The first direct line of evidence that links myotonia congenita to an abnormality in the permeability of the muscle fiber membrane for Cl^- was provided in 1966 when Lipicky and Bryant (1966) demonstrated a reduced $^{36}Cl^-$ efflux from muscles from myotonic goats as compared with that observed from healthy muscles. They later substantiated these findings by direct electrophysiological measurements of reduced G_{Cl} in patients with myotonia congenita (Lipicky et al., 1971). Furthermore, Adrian and Bryant (1974) were able to experimentally

induce myotonic after-discharges and slow relaxation in isolated muscles from healthy goats by simply substituting Cl^- with nonpermeable anions or by blocking the Cl^- permeability. The collective work of Bryant and colleagues revealed an inverse relation between muscle excitability and G_{Cl} and, furthermore, demonstrated that myotonia congenita is associated with very low G_{Cl}. However, their studies did not provide a molecular explanation for the markedly reduced G_{Cl} in the myotonic muscle.

During 1979–1982, Christopher Miller and colleagues first showed that the electroplax membrane from the torpedo electric organ contained an unknown voltage-dependent anion channel (White and Miller, 1979; Miller and White, 1980). From single-channel recordings, it was shown that the channel occupied three conductance states: a zero-conductance state, an intermediate conductance state, and a maximal conductance state (Miller, 1982). The most notable feature of these observations was that the intermediate conductance state was exactly half the maximal conductance state. With impressive foresight, Miller (1982) suggested that the molecular structure of this anion channel had to contain two identical ion-conducting pathways that operated independently during channel bursting. This apparent "double-barreled" torpedo anion channel was cloned in 1990 and became known as ClC-0 (Jentsch et al., 1990). From then on, it soon became evident that Cl^- transporting membrane proteins with similar structures are expressed in different organs across a wide range of organisms (Jentsch et al., 2002; Stauber et al., 2012). Steinmeyer et al. (1991) described and cloned the muscle-specific Cl^- channel in rat that became known as ClC-1. This channel was also found in human muscle, and when the human ClC-1 gene was cloned in 1992, mutations in the ClC-1 gene were discovered to underlie the reduced G_{Cl} and pathological hyperexcitability in myotonia congenita patients (Koch et al., 1992).

Is ClC-1 the only Cl⁻ channel in skeletal muscle membrane?

Several studies report an almost complete absence of G_{Cl} in muscle fibers from mice, goats, and humans with myotonia congenita. This demonstrates that ClC-1 is the major Cl^- channel in skeletal muscle (Lipicky et al., 1971; Mehrke et al., 1988; Bryant and Conte-Camerino, 1991). Nevertheless, several studies from myotubes report electrophysiological observations of Cl^- ion channels with single-channel conductance and kinetic characteristics that differ considerably from those observed with ClC-1. Thus, Blatz and Magleby (1983) reported that in rat myotubes a large-conductance Cl^- channel was occasionally observed when inside-out patches were maintained at 0 mV. This channel had single-channel conductance of ∼430 pS, clearly different from the 1–1.5 pS of ClC-1 (Pusch et al., 1994). However, a physiological role of this channel was not obvious

because it was observed to inactivate at both positive and negative potentials. When patches from myotubes were clamped at potentials corresponding to the resting membrane potential of skeletal muscle fibers (-85 mV), two additional Cl⁻ channels, a slow and a fast, were observed (Blatz and Magleby, 1985). The molecular identities of these channels are not known, and whether they are present in fully differentiated muscle fibers remains uncertain. It is possible, however, that large-conductance channels with activation at ∼0 mV could have escaped attention because G_M is typically measured at membrane potentials where these channels would be inactive. Hence, it cannot be ruled out completely that these channels are present in muscle fibers and that the conditions leading to their activation just have not yet been discovered. Tentatively, it is possible that large-conductance Cl⁻ channel could open during action potential firing when the membrane potential repetitively traverses the voltage range where these channels would activate. At present, however, molecular and functional evidence of other membrane Cl⁻ channels than ClC-1 in fully differentiated skeletal muscle is lacking.

Structure and biophysical properties of ClC-1 channels
Nine ClC proteins have been found in mammals, of which four ClC proteins are plasma membrane ion channels (ClC-1, ClC-2, ClC-Ka, and ClC-Kb), whereas the remaining five ClC proteins are located intracellularly where they operate as anion–proton exchangers. ClC-1 is almost exclusively expressed in skeletal muscle, and no clear physiological significance of ClC-1 has been found in other tissues (Jentsch et al., 2002; Aromataris and Rychkov, 2006). As all other ClC proteins, ClC-1 is a homodimer with each monomer containing its own ion-conducting pathway or pore. The ClC-1 monomer has both the N- and the C-termini in the cytosol, and it contains 19 α-helical domains (A to S), of which 17 are embedded in the membrane (B to R). The N terminus includes the helix A, and the C terminus contains two cystathionine-β-synthase (CBS) domains and the short S helix. The C-terminal cytoplasmic region with its two CBS domains is notably large. It contains ∼400 out of the total of 991 amino acid residues in the ClC-1 monomer (Tang and Chen, 2011).

ClC-1 is a double-barreled protein with two protopore gates and one common gate. Cloning studies of ClC-0 (Jentsch et al., 1990) and ClC-1 (Steinmeyer et al., 1991) suggested similar structures and functions of the two ClC isoforms. However, detailed information on single-channel kinetics has been considerably more difficult to obtain in ClC-1 than in ClC-0 because ClC-1 single-channel conductance is much smaller than that of ClC-0 (Pusch et al., 1994). Nevertheless, using inside-out patch configuration with low bath pH (6.5)

and an almost equal transmembrane Cl⁻ distribution, Saviane et al. (1999) obtained stable single-channel recordings from ClC-1. The low bath pH was used to increase open probability at negative potentials (inspired by Rychkov et al. [1996]), whereas the equal transmembrane Cl⁻ distribution was used to create a larger driving force for currents at negative voltages. As in ClC-0 recordings, Saviane et al. (1999) noted that single-channel recordings from ClC-1 contained silent periods that were interrupted by bursts of activity during which two conductance states were occupied. The most notable feature of this was again that the smaller conductance state was very close to half the magnitude of the larger conductance state. This suggested that ClC-1 has a similar double-barreled structure as ClC-0. Likewise, the data furthermore indicated that two types of gates—two protopore gates and one common gate—control ClC-1 function. Thus, rapid opening and closing of independent protopore gates could explain channel flickering during bursting periods, whereas the silent periods that separated the bursting could reflect simultaneous closing of both pores by the common gate. Strong evidence of a general double-barreled structure of ClC proteins was provided when the crystal structures of two bacterial ClC proteins were solved (Dutzler et al., 2002, 2003). These structures show that the ClC monomers are hourglass shaped and that each monomer contains an ion transport pathway with three anion (Cl⁻)-binding sites. A negatively charged sidechain of a conserved glutamate residue (E148 in *Escherichia coli* ClC protein) is found to protrude into the ion transport pathway where it can interact with the outermost anion-binding site. Control of ClC channel gating by this glutamate residue was first demonstrated by removal of the negative charge on the glutamate side chain in ClC-0 (Dutzler et al., 2003). The selectivity filter is positioned roughly in the middle of the membrane, and ion permeation is believed to proceed in a one-file motion with ions jumping along the three anion-binding sites. Importantly, these structural elements of the bacterial ClC proteins are composed of amino acid sequences that are highly conserved in all ClC proteins, and it is now generally accepted that homodimeric ClC proteins have two ion conducting or transporting pathways irrespective of whether being ion channels or transporters.

The protopore gating. To determine the role of the conserved glutamate (E232) for ClC-1 gating, Cederholm et al. (2010) replaced E232 with a neutral glutamine residue and expressed the channel in HEK 293 cells. Because this replacement was shown to completely abolish protopore gating, it is now generally accepted that protopore gating of ClC-1 involves the negatively charged carboxylic group of E232. More specifically, the carboxylic side chain of E232 is believed to compete with Cl⁻ for the outermost Cl⁻-binding site in the ion

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permeation pathway of ClC-1, and when occupied by E232, it effectively prevents the flow of Cl^- ions through the protopore. This competition between Cl^- and the glutamate residue turned out to be important for the voltage dependence of protopore gating. Unlike the voltage dependence of cation channels, which is conferred by specific structural parts of the channel that enforce configurational changes in the channel structure when moving in response to changes in the transmembrane field (Sigworth, 1994), it has not been possible to identify structural parts of the ClC proteins that act as specific voltage sensors. Instead, it is believed that protopore gating arises from a close coupling between channel permeation and gating in such a way that depolarization favors that a Cl^- ion binds to the external anion binding instead of E232, whereas hyperpolarization favors E232 binding. This mechanism will confer voltage dependence to the channel as it only conducts when the outermost anion-binding site is occupied by Cl^-. This mechanism of gating was suggested on the basis of observations in both ClC-0 and ClC-1 of protopore gating being dramatically reduced when external but not internal Cl^- was lowered (Pusch et al., 1995; Chen and Miller 1996; Rychkov et al., 1996).

Protopore gating in ClC-1 is fast with activation time constants at room temperature <1 ms for positive voltages (Accardi and Pusch, 2000). This means that the gating most likely is sufficiently fast for the channel to activate during the time course of a skeletal muscle action potential. Such activation would facilitate outward, repolarizing current that can reduce the action potential upstroke velocity and peak potential. However, the inward rectifying properties of ClC-1 currents may reduce the importance of ClC-1 currents in shaping the action potential (Lueck et al., 2007). Nonetheless, it can be speculated that some ClC-1 channels open during action potential firing but rapidly deactivate upon cessation of firing. Such voltage-dependent activation of ClC-1 by action potentials would facilitate an increased membrane conductance for Cl^- during trains of closely coupled action potentials. In support of this hypothesis, a study using HEK 293 cells shows that the ClC-1 current at −80 mV increases by ∼70% after imposing 30 short (5 ms) voltage pulses from −80 to 30 mV at 50 Hz (Tsujino et al., 2011). However, whether a similar activity-dependent activation of ClC-1 develops in native tissue during action potential trains remains to be determined.

The common gate. The understanding of the molecular mechanisms underlying common gating has been lacking behind the faster developing understanding of protopore gating. Nevertheless, ClC-1 mutations observed in myotonia congenita patients and mutagenesis studies have shown that common gating can be affected by structural elements of ClC-1 that are far apart. This

suggests that common gating involves long-range interactions between structural elements of ClC-1, including the dimer interface and the CBS domains (Pusch 2002; Lossin and George, 2008; Tang and Chen, 2011). Recently, Bennetts and Parker (2013) used a model that had been proposed for describing the molecular mechanics in prokaryotic Cl^-/H^+ exchange (Feng et al., 2010) as a starting point for exploring common gating of ClC-1 channels. In particular, they noted that the molecular mechanisms of prokaryotic Cl^-/H^+ exchange involved the generation of a hydrogen bond between an extracellular glutamate and an intracellular tyrosine. This was of interest because common gating in both ClC-0 and ClC-1 had previously been associated with a transient transport of protons, a finding that had prompted the notion that these channels in fact are "broken transporters" (Picollo and Pusch, 2005; Lísal and Maduke, 2008). Several lines of evidence suggest the formation of a hydrogen bond during common gating. Thus, most residues and structural elements that are responsible for prokaryotic Cl^-/H^+ exchange are conserved in ClC-1 channels, including the glutamate residue (E232 in ClC-1) on the extracellular side of the pore, which is involved in protopore gating. Also conserved is a tyrosine residue on the intracellular side of the pore (Y578 in ClC-1), which is believed to act as an intracellular gate in the transporter (Miller, 2006). Importance of E232 also in common gating is supported by the findings of Cederholm et al. (2010), who showed that neutralizing E232 by substituting it with glutamine abolishes not only protopore gating but also affects common gating, leaving a completely open channel. Building on these observations, Bennetts and Parker (2013) proposed that common gating involves a channel state in which E232 and Y578 both approach the center anion-binding site from the extra- and intracellular sides, respectively, and here form a hydrogen bond that connects the carboxyl group of E232 to the phenolic-hydroxyl group of Y578. They confirmed the hypothesis in a series of experiments. First, a range of mutations of Y578 caused hyperpolarizing shifts of the voltage dependency of the common gate and clearly increased its residual open probability at very negative membrane voltages. Second, extracellular Zn^{2+} is known to inhibit ClC-1 by interacting with common gating (Chen, 1998; Duffield et al., 2005). Bennetts and Parker (2013) showed that Zn^{2+} did not affect Y578 mutants that were unable to form hydrogen bonds between E232 and the Y578 position. Third, a range of mutations in CBS2 are known to affect common gating although this part of the channel structure is clearly not membrane imbedded and therefore not sensitive to voltage per se. Instead, CBS2 appears to have an allosteric effect on the channels and by this route affects common gating (Estévez et al., 2004; Bennetts et al., 2005). This interaction of CBS2 on common gating is furthermore sensitive to

a range of metabolites (Bennetts et al., 2005, 2007, 2012; Tseng et al., 2007, 2011; Zhang et al., 2008). These metabolites therefore gain important physiological effects on ClC-1 function and G_{Cl} in muscle fibers via altered common gating as described in detail below (Adenosine nucleotide regulation of ClC-1 common gating). When Bennetts and Parker (2013) mutated Y578, it abolished NAD^+-mediated gating. This suggested that the R helix containing Y578 at its N-terminal end is pivotal for linking CBS2 action to common gating. Collectively, the study by Bennetts and Parker (2013) gives strong evidence that Y578 plays a critical role in common gating, and it is compatible with this gating involving a hydrogen bond between E232 and Y578, akin to the proposed molecular mechanisms underlying Cl^-/H transport in the transporter cousins of the ClC channels. What still needs clarification is how the common gating affects both pores simultaneously across the intersubunit interface, and the voltage dependence of this mechanism is not yet clear.

The kinetics of common gating of ClC-1 is ~10-times slower than protopore gating operating with time constants of ~30 and 10 ms at −80 and 0 mV, respectively (Accardi and Pusch, 2000). Because this is much slower than the time course of the skeletal muscle action potential, it appears that this gate is little altered during action potential firing. Rather, it appears plausible that the slow gate is predominantly regulated through signaling pathways or small molecules like ATP that have allosteric effects on the gate.

Adenosine nucleotide binding between CBS domains 1 and 2 is central for ClC-1 channel function. Each ClC-1 subunit contains two CBS domains at the cytosolic C-terminal tail. These domains are sequence motifs of ~60 amino acids. This type of domain was first identified in the CBS protein. CBS domains are highly conserved, being found from archaea to humans (Bateman, 1997), and several of the CBS-bearing proteins are involved in energy sensing in cells (Scott et al., 2004), including AMP kinase and IMP dehydrogenase (Bowne et al., 2002). The physiological importance of the CBS domains is evident from CBS domain mutations underlying several diseases, including Wolf-Parkinson-White syndrome (AMP kinase) and retinitis pigmentosa (IMP dehydrogenase; Ignoul and Eggermont, 2005). CBS domains are present in all eukaryotic ClC proteins (Estévez et al., 2004), and mutations in these domains are associated with a range of diseases, including myotonia congenita (ClC-1; Pusch, 2002), idiopathic generalized epilepsy (ClC-2; Kleefuss-Lie et al., 2009), hypercalciuric nephrolithiasis (ClC-5; Lloyd et al., 1996), and Bartter syndrome (ClC-KB; Konrad et al., 2000).

Binding of ATP to ClC proteins was confirmed when the structure of the C-terminal domain of ClC-5 was solved with ATP bound in a pocket between CBS1 and 2

(Meyer et al., 2007). Although ATP appears to increase Cl^- transport in ClC-5, it has an inhibitory effect on ClC-1. Nevertheless, the structure of ClC-5 has proven useful for building homology models of the CBS domains in ClC-1, and such models allowed identification of important residues for the adenosine nucleotide binding to ClC-1. Residues have thus been identified where their mutations can either abolish ATP modulation of ClC-1 (V634A and E865A) or greatly increase the ATP sensitivity (V643W; Tseng et al., 2011). Several other important residues for metabolite regulation of ClC-1 function have been found, including T636 and P638 from CBS1 and H847 and L848 from CBS2 (Bennetts et al., 2005). As described above (The common gate), CBS domains are expected to exert important influence on common gating of ClC-1 through interaction with Y578 at the N-terminal end of the R helix. This metabolite binding between the CBS domains appears to be a key element in the normal physiological regulation of ClC-1 in active muscle with adenosine metabolites (ATP, ADP, and AMP), shifting the activation curve of common gate in the depolarizing direction and effectively lowering the ClC-1 channel activity at normal resting membrane potentials (Bennetts et al., 2005).

Physiological parameters that affect ClC-1 function

The ATP consumption in muscle fibers can increase >100-fold when muscles are recruited to accommodate intensive physical activity. Although counterbalanced by rapid and extensive ATP resynthesis within the fibers via different cellular pathways, intensive exercise does lead to considerable changes in the cytosolic milieu, including marked accumulation of inorganic phosphate (Pi), acidification, lactate accumulation, and decline in ATP levels (Karatzaferi et al., 2001). The cellular changes depend on the intensity and duration of the exercise and on fiber type, with fast-twitch fibers showing much more dramatic reductions in ATP. Furthermore, in fast-twitch fibers, ATP molecules can become completely degraded through ADP and AMP to IMP via their fiber type–specific high AMP deaminase activity during contractile activity: Karatzaferi et al. (2001) thus observed that in fast-twitch human fibers, the postexercise ATP level was reduced to ~20% of its resting level with a corresponding rise in IMP. Within 90 s of rest, the ATP level had returned to 68% of its resting level and IMP had correspondingly dropped (Karatzaferi et al., 2001). It is believed that the conversion of adenosine nucleotides to IMP is important for maintaining a sufficient free energy yield during ATP hydrolysis (Sahlin et al., 1978; Hancock et al., 2006). In slow-twitch fibers, both the degradation of ATP and the accumulation of IMP during activity are much less. Thus, in the Karatzaferi et al. (2001) study of human muscle, ATP only declined by 25% in slow-twitch fibers, again showing up as IMP accumulation. Similar observations were made in rodent

muscle: In rat fast-twitch muscle, IMP accumulates extensively during contractile activity, whereas in slow-twitch muscle IMP hardly rises (Meyer et al., 1980). However, IMP can be observed to rise dramatically also in slow-twitch muscle under unphysiological or pathological conditions such as with ischemia and in the presence of glycolytic inhibitors (Dudley and Terjung, 1985; Whitlock and Terjung, 1987).

Many steps in the excitation–contraction coupling of skeletal muscle are sensitive to small molecules that either accumulate or decline in working muscles. This has led to multiple theories on the etiology of muscle fatigue that rarely are mutually exclusive (Allen et al., 2008): To mention a few, this includes shifts in Ca^{2+} sensitivity and maximum force of the contractile apparatus in response to accumulation of Pi (Allen and Trajanovska, 2012), reduced open probability of SR Ca^{2+} release channels (RyR1) caused by Mg^{2+} accumulation and decline in ATP (Dutka and Lamb, 2004), and reduced function and possibly leak through the SR Ca^{2+} ATPase (Macdonald and Stephenson, 2006). It has further been suggested that extensive accumulation of Pi could result in Ca^{2+} and Pi precipitating in the SR lumen, further compromising the SR Ca^{2+} release capacity (Allen and Westerblad, 2001). Multiple surface ion channels are sensitive to the metabolic state of the cells expressing them. The K_{ATP} channel is the archetypical ion channel that links the metabolic state of the cells to the cellular excitability (Noma, 1983). In skeletal muscle, K_{ATP} channel activation is known to reduce the action potential overshoot (Light et al., 1994; Gong et al., 2003). More recently, ClC-1 channels have joined the repertoire of ion channels that are sensitive to the metabolic state of cells (Bennetts et al., 2005), and as discussed below (Acute regulation of ClC-1 in the active muscle fiber), this regulation appears to be central for ClC-1 function and muscle excitability in working muscle (Pedersen et al., 2009a,b; Riisager et al., 2014).

Adenosine nucleotide regulation of ClC-1 common gating. In 2005, Bennetts et al. (2005) reported that adenosine nucleotides greatly affect common gating of ClC-1 channels. It was shown that for concentrations of ATP or AMP between 2 and 5 mM, the common gating remained rather constant. In contrast, if the concentration of ATP or AMP was lowered <2 mM, the voltage dependency of gating of the common gate was shifted in the hyperpolarizing direction. This shift was 15 and 30 mV for reductions of ATP or AMP to 1 or 0.5 mM, respectively. In contrast to the adenosine nucleotides, adenosine, adenine, and IMP did not affect ClC-1 function. Indeed, IMP appears to be completely inert for ClC-1 function, as experiments with the combination of ATP and IMP gave similar results as ATP alone (Bennetts et al., 2005). These observations led Bennetts et al. (2005) to suggest that modulation of ClC-1 by adenosine

nucleotides could be involved in fatigue of working muscle. They reasoned that when ATP is broken down to IMP, as especially occurs in exhausted fast-twitch muscle, the common gate would be shifted in the hyperpolarizing direction, leading to marked channel activation. The consequent large increase of G_{Cl} could effectively shut off action potential excitation and propagation in the muscle fibers. No direct evidence for ClC-1 activation in muscle was, however, provided in this study given that all experiments were conducted with ClC-1 in expression systems.

Acidification inhibits ClC-1 via increased ATP sensitivity: A redox-dependent mechanism. Inhibition of G_{Cl} in frog muscle fibers by acidification was first observed by Hutter and Warner (1967a,b) and later confirmed in mammalian muscle (Palade and Barchi, 1977; Pedersen et al., 2005). In rat soleus muscles, it was shown that intracellular acidification to pH 6.8 caused a 35% reduction of G_{Cl}, whereas a similar extracellular acidification had no effect on G_{Cl} (de Paoli et al., 2007). However, this well-established reduction of G_{Cl} in native tissue with intracellular acidification was not straightforward to explain at the molecular level when the effect of acidification on ClC-1 function was explored in expression systems. Using expressed ClC-1, it was first observed that extracellular acidification increased the activity of ClC-1, whereas intracellular acidification had less of an effect (Rychkov et al., 1996). Such findings could obviously not explain the observed effects of pH on G_{Cl} in native tissue. Subsequent experiments in expression systems revealed that the inhibition of ClC-1 by intracellular acidification requires the presence of ATP. Thus, Bennetts et al. (2007) and Tseng et al. (2007) showed that intracellular acidification enhances the ATP sensitivity of ClC-1. Zifarelli and Pusch (2008) were subsequently unable to reproduce the findings of ATP inhibition of ClC-1 at any pH. These apparently conflicting observations were finally explained when Zhang et al. (2008) demonstrated that the ATP inhibition of ClC-1 depends on pH and the redox state of the cell. They showed that oxidation of ClC-1, which could occur in the inside-out patch recording that was used by Zifarelli and Pusch (2008), leads to loss of the ClC-1 sensitivity to ATP. They further showed that ClC-1 remains sensitive to ATP in inside-out preparations if reducing agents were included in the experimental solutions. In the expression systems, the ClC-1 inhibition is maximal at ATP concentrations >2–3 mM (pH 7.2). Nevertheless, acidification still reduces G_{Cl} in resting muscle fibers and in skinned muscle fibers despite that ATP concentrations were >5 mM. This could suggest that the ATP sensitivity of ClC-1 is considerably reduced in native tissue compared with heterologously expressed ClC-1 or that yet another inhibitory effect of acidification on ClC-1 channels exists in native tissue that remains to be identified.

Collectively, these studies demonstrate that physiological phenomena can be challenging to deduce using the reductionist approach of expression systems. Nevertheless, the studies have provided a detailed and important understanding of ClC-1 regulation and appear to have reached the conclusion that reduced G_{Cl} in native tissue in response to intracellular acidification reflects increased ATP inhibition of ClC-1. The inhibition of ClC-1 by ATP is caused by a depolarizing shift of the activation curve of the common gate, leading to a larger fraction of ClC-1 channels being in the closed configuration at the resting membrane potential. This pH-sensitive ATP inhibition furthermore requires a well-maintained redox state of the cell. It can therefore be speculated that oxidative stress of muscle fibers may reduce ClC-1 inhibition by ATP and introduce ClC-1 channel activation with ensuing loss of muscle fiber excitability.

PKC. Inspired by the observation of PKC being able to modulate Cl^- channel function in neurons, Brinkmeier and Jockusch (1987) showed that PKC activation with different phorbol esters caused a dose-dependent and reversible reduction of G_{Cl} in mouse muscle fibers. They observed that with sufficiently long exposure times and high concentrations of phorbol esters, G_{Cl} could be reduced <20% of its control level, and at this degree of G_{Cl} reduction, myotonic action potentials started to appear. Similar reduction of G_{Cl} with phorbol esters was later observed in frog (Tricarico et al., 1993), rat (Tricarico et al., 1991), and goat muscle (Bryant and Conte-Camerino, 1991). Subsequently, it has been demonstrated that PKC-mediated inhibition of G_{Cl} is involved in reduction of G_{Cl} with niflumic acid (Liantonio et al., 2007), statins (Pierno et al., 2009), and muscle disuse (Pierno et al., 2007). Common to these findings is that a small rise in the cytosolic Ca^{2+} is the activator of PKC. Slightly elevated resting cytosolic Ca^{2+} in rat slow-twitch muscle compared with rat fast-twitch muscle also appears to cause PKC-mediated G_{Cl} reduction even at rest in slow-twitch muscle (Pierno et al., 2007; Pedersen et al., 2009b).

The molecular mechanism by which PKC inhibits G_{Cl} in native tissue was initially explored with ClC-1 expressed in HEK 293 cells (Rosenbohm et al., 1999). It was observed that phorbol esters reduced ClC-1 currents without affecting voltage dependence and open probability of the channels. This suggested that PKC activation affects the function of active channels by inhibiting ion permeation, effectively reducing single-channel conductance. However, more recent findings on ClC-1 channels expressed in *Xenopus* oocytes reported that the ClC-1 activation curve was shifted to more depolarized membrane potentials when exposed to phorbol esters (Hsiao et al., 2010; Riisager et al., 2016). In the latter study, it was additionally shown that the PKC activation changed the voltage-dependent open probability through alterations of both the protopore

and common gating mechanisms, whereas the maximum current was not affected. The exact reason for the apparent discrepancies between these findings is not clear at this moment but might involve differences in channel expression systems and recording techniques used in the studies. The PKC phosphorylation site on ClC-1 has in one study been suggested to involve Thr891-Ser892-Thr893 in the C-terminal part of the protein (Hsiao et al., 2010).

Although PKC has been known for a long time to be a potent regulator of ClC-1 function and G_{Cl} in native tissue, it is only recently that the physiological role of this regulation in active muscle has become clear. As described in detail below (Acute regulation of ClC-1 in the active muscle fiber), onset of repeated firing of action potentials triggers a PKC-dependent reduction in G_{Cl} (Pedersen et al., 2009a,b; Riisager et al., 2016). This suggests that PKC-dependent ClC-1 inhibition has the physiological role of preserving muscle excitability during muscle activity.

Subcellular distribution of ClC-1 channels in skeletal muscle

The subcellular distribution of ClC-1 channels between the sarcolemma and the t-system has been intensely debated (Pugh, 2011). Most functional studies provide convincing evidence that t-tubular membranes of both amphibian and mammalian muscle fibers have a rather large G_{Cl}. In contrast, some immunohistochemical data suggest that ClC-1 is absent from the t-system. Two types of functional studies have been performed to determine the presence and role of Cl^- channels in the t-system.

First, mechanical skinning of muscle fibers provides a unique technique in which the t-system excitability can be studied in isolation from the sarcolemmal membrane: Intact rat muscles (extensor digitorum longus [EDL]) are placed in paraffin oil, and with a set of fine forceps, the experimenter holds onto the surface of a single fiber and gently pulls back a superficial layer of the fiber. As this layer is separated from the rest of the fiber, the sarcolemma rolls back, creating direct access to the intracellular compartment. This procedure is referred to as mechanically skinning (Posterino et al., 2000). An inside-out fiber segment is the result, and this can then be tied at both ends and attached to a force transducer. Remarkably, the t-tubular system seals off, and when transferred to solutions that mimic the normal intracellular milieu, the fibers twitch and tetanize when stimulated electrically. Having lost the sarcolemma membrane, such responses to electrical stimulation reflect the generation of action potentials in the sealed t-tubular membrane tubes, as confirmed by the absence of contractions in fibers if the voltage-gated Na^+ channels are blocked by tetrodotoxin (Posterino et al., 2000). Although the mechanically skinned fiber gives indirect data on G_{Cl}, as it has to be interpreted from measurements of contractile force, it has provided ample and clear-cut evidence that

the t-system membrane has a substantial G_{Cl}. Hence, these preparations depolarize and rapidly contract when exposed to an intracellular solution that contains a high Cl^- concentration (Lamb and Stephenson, 1990; Coonan and Lamb, 1998). Because the high internal Cl^- concentration is only able to depolarize the t-system and cause voltage sensor activation if the t-system membranes have a substantial G_{Cl}, this represents strong albeit indirect evidence for the presence of a t-system Cl^- ion channel. Other experiments have shown that the excitability of the skinned fiber increases when the fiber segments are exposed to the ClC-1 inhibitor 9-AC and when exposed to phorbol esters (Dutka et al., 2008) that stimulate PKC-mediated ClC-1 inhibition. Acidification and lactate were also shown to increase the excitability of both intact muscle (Pedersen et al., 2005; de Paoli et al., 2010) and mechanically skinned fibers (Pedersen et al., 2004; de Paoli et al., 2010), and this only occurred in the presence of Cl^-. Collectively, the skinned fiber experiments give strong evidence that the t-system does have a substantial G_{Cl} and the pharmacological profile of this tubular G_{Cl} appears identical to G_{Cl} of intact fibers. This strongly argues that ClC-1 is the channel responsible also for t-tubular G_{Cl}.

Second, electrophysiological experiments have typically measured G_{Cl} in fibers before and after exposure to an osmotic shock that detaches at least parts of the t-system in the fibers (Eisenberg and Gage, 1967). The success of such detubulation after the osmotic shock is typically evaluated from the reduction in muscle fiber membrane capacitance. Although detubulation would appear to be the obvious approach to determine whether ClC-1 channels are located only in the sarcolemma or also in the t-system, divergent results with detubulation have been reported: Some studies with rat muscle present data compatible with a substantial G_{Cl} in the t-system, as reflected by loss of G_{Cl} with detubulation (Palade and Barchi, 1977; Dulhunty 1979). In contrast, other studies using frog and mouse muscles suggest that ClC-1 is only present in the sarcolemma because, in these studies, detubulation left G_{Cl} virtually unaffected (Eisenberg and Gage, 1969; Lueck et al., 2010). Recently, voltage clamping to measure G_{Cl} in isolated mouse fibers was performed in combination with recordings of t-tubular voltage as determined by potentiometric dyes (DiFranco et al., 2011). The experimental findings with this elegant approach were compared with simulations using a radial cable model of the t-system, and it was shown that the experimental data were best (not to say only) reproduced in silico when similar G_{Cl} were allocated to the sarcolemma and the t-system.

The caveat in unequivocally allocating ClC-1 to the t-system is the immunohistochemical data in which staining with ClC-1 antibodies has thus far only been able to show the presence of ClC-1 in the sarcolemma (Gurnett et al., 1995; Papponen et al., 2005). However, considerable

concerns regarding these techniques have been raised (Lamb et al., 2011). Another imaging approach has been to express GFP- or YFP-tagged ClC-1 channels in mouse muscle fibers in vivo by electroporation or by adenovirus transfection and then determine their subcellular distribution. Again, divergent results were presented: Lueck et al. (2010) expressed GFP-tagged ClC-1 channels in fibers from dystrophic mice and arrived at the conclusion that these channels only trafficked to the sarcolemma, whereas DiFranco et al. (2011) showed that expressed YFP-tagged ClC-1 channels also traffic to the t-system in healthy muscle fibers from mice.

Thus, although the majority of studies on the subcellular ClC-1 distribution suggest that these channels are present in the t-system, some studies do present solid evidence arguing the opposite. This unresolved issue begs the question of whether the subcellular distribution of ClC-1 can vary under various conditions. A study by Papponen et al. (2005) indeed suggested that ClC-1 channels can become internalized when muscle fibers are isolated and, interestingly, the channels return to the sarcolemma when given the nonspecific kinase inhibitor staurosporine or when stimulated electrically. Whether alteration in trafficking may explain the different observations in different laboratories could be explored further.

The distribution of chloride and its relevance for muscle excitability

The extent and physiological role of active Cl^- transport in skeletal muscle is not completely clarified. Typically, active transport of Cl^- has in skeletal muscle been studied by measuring the resting membrane potential or intracellular Cl^- activity when blocking Cl^- channels or substituting Cl^- with impermeable anions.

Several studies have reported that Cl^- substitution or Cl^- channel blockade cause a hyperpolarization, whereas other studies found that these treatments did not affect the resting potential. Thus, substituting Cl^- with isethionate was reported to cause a 2.5–12-mV hyperpolarization in mouse soleus muscle (van Emst et al., 2004). In this study, the hyperpolarization upon removal of Cl^- depended inversely on the extracellular K^+ concentration with the hyperpolarization being 12 mV at 5 mM K^+ and 2.5 mV at 12.5 mM K^+, respectively. The larger hyperpolarization with removal of Cl^- at low/normal extracellular K^+ was interpreted to reflect that an active Cl^- transport mechanism, suggested to be NaK2Cl cotransport, was only detectable at normal extracellular K^+, under which conditions it elevates intracellular Cl^- above that expected from passive distribution. Blocking Cl^- channels with 9-AC was similarly found to hyperpolarize the resting membrane potential of rat and mouse lumbrical muscle (Harris and Betz, 1987; van Mil et al., 1997; Geukes Foppen et al., 2002). In contrast, multiple studies have reported that neither Cl^-

channel blockade nor Cl⁻ substitution affected the resting membrane potential in frog, rodent, or human muscles (Hodgkin and Horowicz, 1959; McCaig and Leader, 1984; Kwieciñaski et al., 1988).

In 1967, Hutter and Warner produced a series of three papers on inhibitory effects of extracellular acidification on G_{Cl} in frog muscle (Hutter and Warner, 1967a,b,c). Among their observations was a rise in fiber G_{Cl} and a transient depolarization when acidified fibers with low G_{Cl} were exposed to alkaline solution (Hutter and Warner, 1967a). They demonstrated that this transient depolarization with alkalinization was caused by Cl⁻ efflux from the fibers, and they suggested that during the acidic preincubation, which was associated with low G_{Cl}, an active transport mechanism had increased intracellular Cl⁻ above the concentration expected from passive distribution. With subsequent alkalinization, G_{Cl} rose and this led to a transient Cl⁻ efflux whereby E_{Cl} eventually settled close to the resting membrane potential as expected from a completely passive distribution of Cl⁻. These findings pointed out that active Cl⁻ transport can be hard to detect experimentally when short-circuited by a large G_{Cl}. In agreement with this, Bolton and Vaughan-Jones (1977) used Cl⁻-sensitive microelectrodes to confirm that active Cl⁻ accumulation is present in frog muscle fibers but G_{Cl} had to be reduced by acidification for it to be revealed. Similarly, in rat lumbricalis muscle, Cl⁻ accumulation was observed when G_{Cl} was reduced by 9-AC (Harris and Betz, 1987; Aickin et al., 1989). It was further demonstrated that denervation of rat lumbricalis muscle fibers was associated with reduction in G_{Cl} and a marked rise in intracellular Cl⁻ (Harris and Betz, 1987). Although the Cl⁻-sensitive microelectrodes have been subject to some serious criticism (McCaig and Leader, 1984; Chao and Armstrong, 1987), the collective observations suggest that G_{Cl} reduction can lead to intracellular Cl⁻ accumulation. These findings present strong evidence for the presence of some degree of active Cl⁻ transport in muscle, but the physiological role of this transport system in working muscle including volume regulation is probably limited (Usher-Smith et al., 2009; Lindinger et al., 2011).

The cellular transport mechanism responsible for active Cl⁻ transport in muscle has been studied by determining the effects of furosemide, bumetanide, and ion substitution on membrane potential, intracellular Cl⁻ activity, and ion fluxes. Multiple studies report that bumetanide and/or furosemide induce a hyperpolarization (Harris and Betz, 1987; van Mil et al., 1997), and in one of these studies, furosemide caused the intracellular Cl⁻ activity to decline to a level expected from passive distribution (Harris and Betz, 1987). Secondary active transport of Cl⁻ was further supported by a passive distribution of Cl⁻ in Na⁺-free solution (Aickin et al., 1989). K⁺-free solutions were also argued to remove

any indications of active Cl⁻ transport, but these experiments were greatly biased by the depolarized membrane potential (Aickin et al., 1989). Although these observations are compatible with the NaK2Cl cotransporter being responsible for active transport of Cl⁻ in skeletal muscle, a study by Dørup and Clausen (1996) showed that bumetanide-sensitive ²²Na⁺ influx in rat soleus and EDL muscles was not associated with a corresponding bumetanide-sensitive ⁴²K⁺ influx. These findings were suggested to reflect that the main active Cl⁻ transport in these muscles was not via the NaK2Cl cotransporter but rather through an NaCl cotransporter. However, in contrast to the demonstration of the presence of the NaK2Cl cotransporter in rat soleus muscle at the mRNA and protein levels (Wong et al., 1999), the molecular evidence for the presence of the NaCl cotransporter in skeletal muscle is still lacking. Perhaps for this reason, the general notion appears to be that active Cl⁻ transport in muscle is mainly via the NaK2Cl cotransporter.

Active Cl⁻ transport in skeletal muscle is thus associated with conflicting experimental observations. Some studies show that it affects the resting membrane potential, whereas others report that Cl⁻ has little effect on the resting membrane potential, and conflicting evidence exists on whether the transporter is the NaK2Cl⁻ cotransporter or the NaCl cotransporter. Some of these apparent discrepancies may be reconciled by considering the sensitivity of the transport to extracellular tonicity. Thus, van Mil et al. (1997) showed that elevating the extracellular tonicity in the range from 266 to 344 mOsm caused a depolarization of ∼11 mV in mouse lumbrical muscle. Although bumetanide and furosemide did not affect the resting membrane potential in hypotonic solutions (266 mOsm), they caused a −3-mV change at normal tonicity (289 mOsm) and up to −12-mV change in hypertonic solution (344 mOsm). The discrepancies between studies of intracellular Cl⁻ activity may similarly be ascribed to the use of extracellular solutions with different tonicities. Dulhunty (1978) found evidence of active Cl⁻ accumulation in mouse EDL, whereas Donaldson and Leader (1984) did not obtain such evidence in the same muscle. This may reflect that the former study used a higher extracellular osmolality (355 vs. 290 mOsm), which activates active Cl⁻ transport (van Mil et al., 1997; Ferenczi et al., 2004). In addition to tonicity, different experimental temperature may explain some of the different findings. Thus, it has been shown that temperature elevation from 27 to 35°C reduces the effect of 9-AC on the resting membrane potential (Geukes Foppen et al., 2002). As suggested by Geukes Foppen et al. (2002), this temperature dependency may explain the larger 9-AC–induced hyperpolarization observed in experiments at 20°C (Aickin et al., 1989) when compared with observations at 35°C (Geukes Foppen et al., 2002).

Summarizing the above, skeletal muscles have the capacity to actively transport Cl⁻ when exposed to hypertonic

conditions and low experimental temperature. To what extent this transport leads to intracellular Cl^- accumulation depends on activity of Cl^- ion channels that counteract Cl^- accumulation by passive Cl^- efflux.

Physiological impact of ClC-1 on muscle excitability and K^+ homeostasis

High G_{Cl} and E_{Cl} close to the membrane potential in resting muscle fibers are the two boundary conditions for the physiological roles of Cl^- channels in skeletal muscle. Hence, whenever the membrane potential deviates from E_{Cl}, a driving force for Cl^- current is created, and the magnitude and physiological impact of this current is scaled by G_{Cl}, which reflects the activity of ClC-1. ClC-1 currents therefore have a short-circuiting and stabilizing effect on the resting membrane potential and thereby reduce the excitability of muscle in at least three different ways.

First, current through ClC-1 channels counteracts action potential excitation and increases the excitatory current required to trigger an action potential (Adrian and Bryant, 1974; Pedersen et al., 2005). This role of ClC-1 channels has typically been studied by inserting two electrodes into the same fibers to determine how much current that needs to be injected to trigger an action potential. The durations of current injection have typically been long (>25 ms) compared with the membrane time constant ($\sim$5 ms), and the membrane potential therefore attains a stable depolarized value during the current injection. During such long current injections, Cl^- channels become the major route for short-circuiting current. It is, however, important to bear in mind that the physiological current flow during neuromuscular transmission is short when compared with the membrane time constant. During such short duration and physiological current flow, the membrane impedance will be dominated by capacitive elements of the membrane and generally be less sensitive to Cl^- current flow (Pedersen et al., 2011). Changes to ClC-1 function will therefore have less physiological effect if current flow is shorter than the membrane time constant. Naturally, if ClC-1 channels activate to such an extent that the membrane time constant becomes shorter than excitatory current flow, the excitability of muscle fibers will be considerably depressed. This occurs in fast-twitch fibers during prolonged activation when a marked activation of ClC-1 and K_{ATP} channels develops (Pedersen et al., 2009a,b, 2011).

Second, because of the narrow lumen of the t-tubules, G_{Cl} attains particular importance for excitation of action potentials in the t-system. Excitation of the t-tubular action potential occurs by circuit currents that flow in front of the propagating sarcolemmal action potential. Because the narrow lumen of the t-tubules exerts a substantial resistance to current flow, the currents involved in charging the t-tubular membrane above action potential threshold must also flow across a substantial resistance positioned in series with the t-tubular membrane impedance. This luminal resistance effectively acts as a low pass filter on the charging of the t-system membrane capacitance, leaving the t-system excitation to be achieved mainly by low-frequency components of the circuit currents (Pedersen et al., 2011). Taking this filtering effect together with the fact that low-frequency currents are more sensitive to G_M than high-frequency currents, the geometry of the muscle fiber with its luminal resistance makes G_{Cl} more important for t-system excitability than for sarcolemmal excitability. Indeed, simulations in Fraser et al. (2011) indicate that it is possible for activation of ClC-1 to render the t-system inexcitable while the sarcolemma continues to excite and propagate action potentials.

Third, during trains of action potentials, current through Cl^- channels reduces both K^+ accumulation in the t-tubular system and the associated depolarization. K^+ rapidly accumulates in the t-system during repeated firing of action potentials (Almers, 1980; Fraser et al., 2011). This occurs because the t-tubular system has a large surface area to volume ratio and repolarizing K^+ currents are directed outward from a high intracellular concentration into the small-volume t-system with a small K^+ concentration. The t-system K^+ accumulation raises E_K and therefore tends to depolarize the fiber during repeated firing. Experimentally, this K^+ accumulation can be observed during trains of sarcolemmal action potentials as a depolarization of the resting membrane potential (rise above dotted line in Fig. 1 A, left). Importantly, both experimental recordings of trains of action potentials and simulations show that the K^+ level in the t-system recovers in less than a second once the action potential firing ceases (Fraser et al., 2011). In contrast to repolarizing K^+ current that flows into a small-volume t-system with a low K^+ concentration, the repolarizing Cl^- current flows from the high concentration in the small-volume t-system into the comparatively large cytosol with a low concentration of Cl^-. This means that in contrast to E_K, there will be very little change to E_{Cl} during repeated firing of action potentials. During action potential trains, t-tubular E_K and E_{Cl} thus diverge, with E_K becoming depolarized while E_{Cl} remain largely constant. The rise in E_K will tend to depolarize the fiber, but because E_{Cl} stays close to the membrane potential of resting muscle fibers, the depolarization will depend nonlinearly on the ratio of G_{Cl} and the membrane conductance to K^+ (G_K). Reductions of G_{Cl} will enhance depolarization, especially if G_{Cl} approaches G_K, which requires G_{Cl} to drop by >60%. Further reduction in G_{Cl} would induce exacerbated depolarization during action potential firing, and this is the basis for developing myotonic action potential firing. The dispersion of E_K and E_{Cl} in the t-system during trains of action potentials means that E_{Cl} clamps the resting membrane potential more negative than E_K, and this generates a driving force for reuptake of t-tubular K^+ via inward rectifying K^+ channels in the t-system. The

significance of the K⁺ reuptake has in simulations been assessed to be of substantial importance for maintenance of t-tubular K⁺ homeostasis (Wallinga et al., 1999).

Because Cl⁻ channels affect skeletal muscle excitability and t-system K⁺ handling in these different ways, the end effect of ClC-1 regulation on muscle function can be hard to predict. On the one hand, inhibition of ClC-1 channels will reduce the current required to trigger an action potential, but this increase in excitability may come at the price of larger t-tubular K⁺ accumulation and larger depolarization of the resting membrane potential during trains of action potentials. The enlarged depolarization could compromise excitability through inactivation of voltage-gated Na⁺ channels. de Paoli et al (2013)

recently showed that the largest contractile endurance of rat muscles was observed if ClC-1 channels were inhibited by ∼70%. If the inhibition was either reduced or larger, the contractile endurance was reduced. On this basis, it was suggested that there exists an optimum degree ClC-1 inhibition for maintenance of contractile endurance. Interestingly, this degree of ClC-1 inhibition is close to what has been observed to occur in both rodent and human muscles when muscles are activated repeatedly (Pedersen et al., 2009a,b; Riisager et al., 2014, 2016).

Acute regulation of ClC-1 in the active muscle fiber

Expression systems have provided unique understanding of the structure–function relationship and the molecular
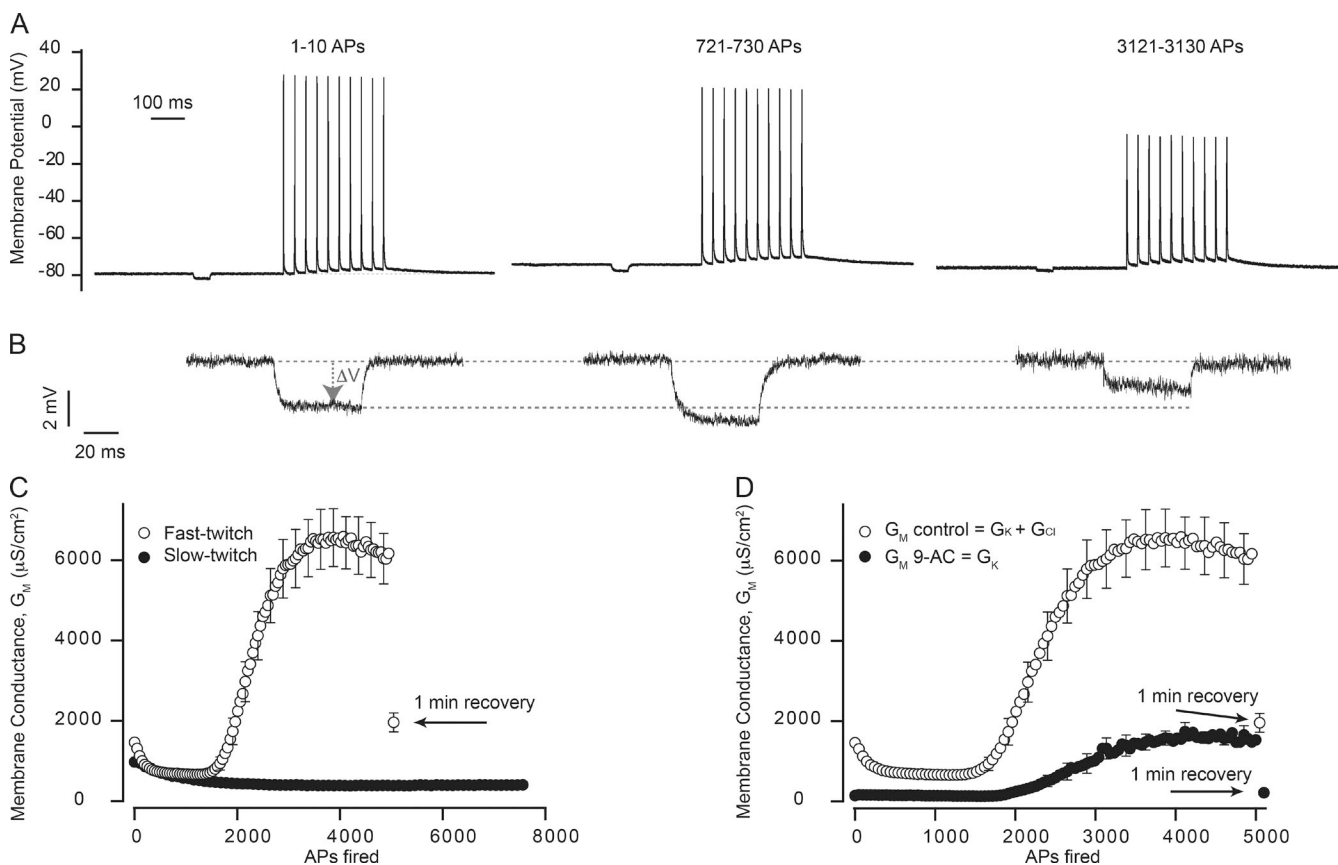
Figure 1. The changes in G_M in active skeletal muscle fibers. Two microelectrodes were inserted into the same muscle fiber: One electrode was used to inject currents, whereas the other electrode recorded the membrane potential. Using this approach, short trains of action potentials (APs) can be repeatedly triggered in the fiber, and in between the trains, G_M can be determined from the membrane potential response (ΔV) to the injection of a 50-ms constant current of small amplitude. (A) Typical recordings from a fast-twitch rat muscle fiber are shown. The dotted line in the first train indicates the resting membrane potential before action potential firing. The depolarized resting membrane potential during action potential firing reflects K⁺ accumulation in the t-system (Fraser et al., 2011). (B) Enlargements of the membrane potential response to the constant current injection are shown. It can be seen that with the onset of action potential firing ΔV became larger. This reflects a reduction in G_M that is caused primarily by PKC-mediated inhibition of ClC-1 channels. With continued activity, ΔV decreased markedly. This reflects activation of both K_{ATP} and ClC-1 Cl⁻ channels. This latter activation of ion channels was associated with clear declines in AP amplitude. (C) Average observations of the G_M changes in fast- and slow-twitch muscle fibers are shown. It can be seen that the rise in G_M with prolonged activity was only observed in fast-twitch muscle fibers. (D) The total G_M in active fast-twitch muscle fibers under control conditions, reflecting the activities of both Cl⁻ and K⁺ channels. Also shown are observations in the presence of 9-AC, which blocks ClC-1. G_M with 9-AC therefore reflects the activity of K⁺ channels alone, and the difference between control G_M and G_M with 9-AC reflects ClC-1 function. Error bars represent SEM values, and to improve clarity of the figure, only every fifth error bar has been included.

mechanism of ClC-1 gating and regulation. Although these approaches will no doubt remain instrumental in future clarification of ClC-1 function and regulation, the physiological significance of findings in expression systems must ultimately be explored in the native tissue of muscle fibers. This is required in part to validate the findings from expression systems and in part to determine how multiple regulatory mechanisms of ClC-1 interact in muscle fibers at rest and during muscle activity. It is nevertheless a challenging task to explore ion channel function in skeletal muscle because muscle fibers have a complex membrane geometry (t-system and sarcolemma) and because the cytosolic milieu of fibers changes substantially during the course of muscle activity. Adding to this, contractile activity complicates measurements of ion channel function with intracellular microelectrodes in action potential firing muscles.

Using inhibitors of myosin heavy chain II (BTS and blebbistatin) it has, however, become possible to almost abolish contractile activity while leaving other steps in excitation–contraction coupling of muscle fibers intact (Macdonald et al., 2005; Pedersen et al., 2009b). When exciting action potentials via one inserted microelectrode and recording the membrane voltage with one or two other electrodes, it has thus become possible to maintain electrodes inserted in muscle fibers while they fire thousands of action potentials (Pedersen et al., 2009a; Riisager et al., 2014, 2016). Using this approach, experiments with intact rat, mouse, and human muscles have revealed that the ion channels that determine G_M are highly regulated during muscle activity and that this regulation primarily occurs through modulation of ClC-1 channel function (Pedersen et al., 2009a,b; Riisager et al., 2016). This technique has opened the possibility to explore regulation of ion channels with subsecond temporal resolution in active muscle fibers, but it should be remembered that it occurs in fibers that have reduced metabolic turnover because the contractile activity has been inhibited.

Thus, as shown by recordings in Fig. 1 (A and B), the onset of action potential firing is associated with an increased deflection to a constant current injection. This increased voltage response reflects a reduction of G_M of up to 70%, and it has been observed in both slow- and fast-twitch muscle of rodents, and more recently in human muscles (Riisager et al., 2016). The reduction in G_M with onset of activity is caused by an inhibition of ClC-1 channels (Pedersen et al., 2009b; de Paoli et al., 2013). The ClC-1 inhibition is predominantly mediated by a Ca^{2+}-sensitive PKC isoform that is activated during the action potential firing. The experimental evidence for this is that both PKC inhibitors and inhibition of SR Ca^{2+} release greatly reduce the activity-induced ClC-1 inhibition. Although the fastest and largest ClC-1 inhibition at the onset of activity appears to be via this PKC-dependent pathway, a slower and less pronounced

G_{Cl} reduction was observed in the presence of PKC inhibitors. Because this slower G_{Cl} inhibition was shown to develop in close temporal association with declining intracellular pH (Pedersen et al., 2009b), it most likely reflected inhibition of ClC-1 by intracellular acidification (Palade and Barchi, 1977; Pedersen et al., 2005) and possibly an effect of the lactate ion per se (de Paoli et al., 2010). The current understanding of the physiological importance of acidification, lactate, and PKC-mediated inhibition of ClC-1 has been developed from two series of experiments. First, it is well known that muscle activity is associated with elevation of extracellular K^+ that at least partly arises from repolarizing currents that flow during repeated action potential firing in muscle fibers. When such K^+ elevation was mimicked by exposing isolated muscle to high extracellular K^+, the force declined because the excitability of the muscle was reduced. Under these conditions of elevated extracellular K^+, further experiments showed that muscle excitability and contractile force can be restored with acidification (Nielsen et al., 2001; Pedersen et al., 2004, 2005), lactate ions (de Paoli et al., 2010), 9-AC–mediated ClC-1 inhibition (Pedersen et al., 2005), and PKC activation (Pedersen et al., 2009b). This suggests that inhibition of ClC-1 at the onset of muscle activity has the physiological effect of preserving muscle excitability in the face of activity-induced elevation in extracellular K^+. Second, a recent study has explored the effect of PKC inhibitors on the force decline during prolonged stimulation in isolated rat muscles (de Paoli et al., 2013). It was demonstrated that force declines faster in the presence of PKC inhibitors but only when Cl^- is present in the bathing solution. This also supports that inhibition of ClC-1 by PKC is important for the ability of muscle fibers to maintain excitability during intense activation. From these two series of experiments, it appears clear that ClC-1 inhibition with the onset of muscle activity represents an important mechanism whereby active muscles preserve their excitability despite that extracellular K^+ rises to concentrations that could potentially compromise excitability. The studies also add support to the developing understanding that muscle acidification and lactate ions have little role in causing muscle fatigue. In contrast, the restoration of muscle excitability and force at elevated extracellular K^+ by acidification and lactate could be considered to reflect protective mechanisms against fatigue (Nielsen et al., 2001).

Slow-twitch fibers can sustain firing of many thousands of action potentials while keeping G_M reduced as the result of PKC-mediated ClC-1 inhibition (Fig. 1 C). In contrast, prolonged action potential firing in fast-twitch fibers can induce a sudden and pronounced rise in G_M (Fig. 1 C). This rise in G_M with prolonged action potential firing activity is rapidly reversed upon cessation of stimulation. The rise in G_M is furthermore closely associated with (a) hyperpolarization of the fibers, (b) a

marked reduction in the action potential waveform, and (c) excitation failures (Pedersen et al., 2009a; Riisager et al., 2014). Recent analysis shows that when the high G_M state develops during prolonged activation, the part of the action potential that is within the voltage range that is capable of triggering SR Ca^{2+} release through voltage sensor activation in the t-system drops dramatically (Riisager et al., 2014). This suggests that SR Ca^{2+} release could be markedly depressed by the high G_M state.

The activity-induced rise in G_M in fast-twitch fibers was shown to reflect cotemporal elevations in both G_{Cl} and G_K (Fig. 1 D). Because experiments with glibenclamide largely prevented the rise in G_K, it is evident that the rise in G_K is caused by K_{ATP} channel activation (Pedersen et al., 2009a). The K_{ATP} channel is the classical example of an ion channel that can sense the metabolic state of cells, thereby representing a link between metabolism and excitability (Flagg et al., 2010). The involvement of K_{ATP} channels in the G_M rise during prolonged action potential firing in fast-twitch fibers therefore suggested that the rise in G_M occurs in response to a substantial reduction in the metabolic state of fiber. Given the recent

evidence that ClC-1 channels are inhibited by ATP and other adenosine nucleotides but not IMP (Bennetts et al., 2005; Tseng et al., 2007), a reduction in the muscle fiber metabolic state leading to IMP accumulation (as occurs specifically in fast-twitch fibers [Karatzaferi et al., 2001]) would be expected to also activate ClC-1. The co-temporality of the activation of K_{ATP} and ClC-1 channels suggests that these channels have similar sensitivities to the metabolic state of the fiber and that these channels join forces in sensing the state of muscle fibers (Fig. 1 D).

Because the high G_M state that develops with prolonged activation in fast-twitch fibers is associated with reduced action potential amplitude (Fig. 1 A, right) and excitation failures, it appears likely that G_M elevation could be a contributing factor to fatigue in fast-twitch muscle. Studies on isolated fast-twitch mouse fibers have repeatedly shown that fatigue develops after firing of ~1,800 action potentials, and this is closely associated with loss of SR Ca^{2+} release (Place et al., 2008). This loss of SR Ca^{2+} release is believed to develop when the metabolic state of the fibers reaches a critical level. Several mechanisms can contribute to the reduced SR
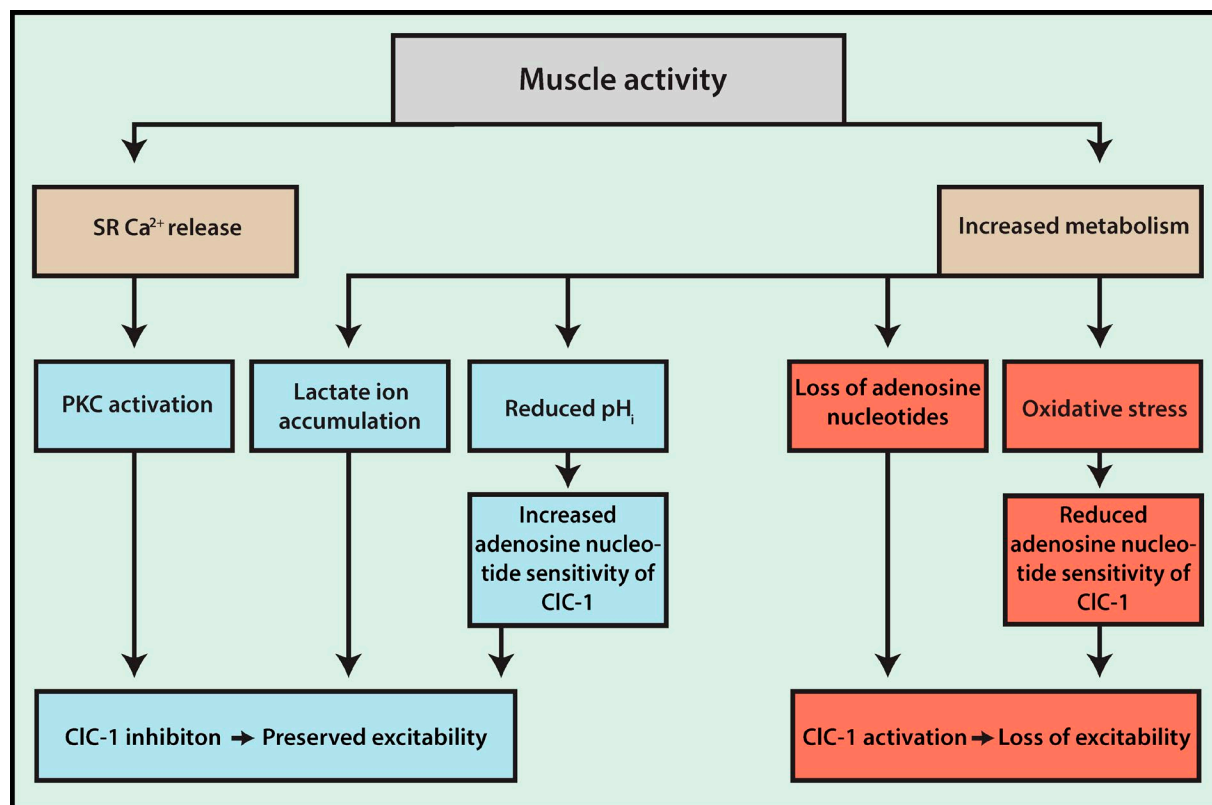
Figure 2. Diagram summarizing physiological regulation of ClC-1 function in active muscle fibers and the consequences for the excitability of the muscle fibers. The figure illustrates that Ca^{2+} released from SR triggers PKC-mediated ClC-1 inhibition. ClC-1 channels are also inhibited via lactate ions and intracellular acidification. The inhibitory action of reduced pH_i on ClC-1 function is at least partly mediated via increased sensitivity for adenosine nucleotides of ClC-1 channels. ClC-1 inhibition represents an important mechanism for the muscle to preserve excitability during repeated action potential firing. Under conditions where ATP consumption exceeds ATP replenishing capacity, the adenosine nucleotides will decline, leading to formation of IMP. Given that IMP is inert on ClC-1 function, the decline in adenosine nucleotide can lead to marked activation of ClC-1. Inhibitory current through ClC-1 channels will thereby increase drastically, and this can shut off muscle excitability and possibly contribute to fatigue.

Ca^{2+} release, including altered ryanodine receptor function (Dutka and Lamb, 2004) and precipitation of Ca^{2+} and Pi in the SR. The rise in G_M in fast-twitch muscle fibers also develops after firing $\sim$1,800 action potentials, a finding that is compatible with G_M elevation and loss of action potential waveform being a contributing factor to the loss of SR Ca^{2+} release during fatigue in fast-twitch muscle. Indeed, given that action potential excitation/propagation and SR Ca^{2+} release are sequential events in EC coupling, it is likely that these mechanisms could additively or possibly synergistically shut down muscle activation when the metabolic state of muscle fiber has dropped to a critical level. An important physiological effect of this could be to avoid further muscle activation that would threaten cellular integrity.

Summary

The understanding of ClC-1 function in skeletal muscle has during the last decade developed from being considered a large leak conductance that simply acts to avoid myotonia to the realization that multiple cellular signals control ClC-1 function during muscle activity (Fig. 2). It has become clear that this ClC-1 regulation plays a central role in the control of excitability of working muscle: With onset of muscle activity, several cellular signals participate in inhibiting ClC-1 channels (Fig. 2, blue boxes), whereby the excitability of active muscle fibers is preserved. During muscle activity, however, the metabolic state of the active fibers gradually declines with loss of adenosine nucleotides, and sufficient energy depletion will promote ClC-1 activation, leading to loss of excitability and possibly fatigue (Fig. 2, red boxes). In addition to controlling the excitability of working muscle, ClC-1 channels also appear to represent a link between muscle excitability and the level of cellular stress, as findings in expression systems suggest that these channels will activate upon fiber oxidation.

Several questions about regulation of ClC-1 channels remain open. (a) The evidence that ClC-1 channels are sensitive to ATP and other adenosine metabolites was obtained with expressed ClC-1, and it remains unknown whether these channels are sensitive to adenosine metabolites in native tissue of muscle fibers. (b) The extent that the t-tubular action potential is affected by ClC-1 and K_{ATP} channel activation remains to be determined. This is clearly important for linking ClC-1 and K_{ATP} channel activation to SR Ca^{2+} release. (c) Finally, it is not known whether ClC-1 and K_{ATP} channel activation is promoted in diseases with altered metabolic state of muscle fibers, but the observation that oxidation reduces ATP sensitivity of ClC-1 does prompt the possibility that ClC-1 activation may be enhanced in disorders with oxidative stress in skeletal muscle.

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