Chloride channels take center stage in a muscular drama

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The generation of action potentials in excitable cells requires selective ion channels that open and close upon changes in membrane potential. Initially, cell excitability was mainly studied in neuronal axons, and in this particular cell compartment, electrical excitability is almost exclusively governed by cation channels. For many years, voltage-dependent anion channels were thought to be of no relevance for cell excitability and considered mere "background" or "leakage" channels involved in housekeeping functions. Skeletal muscle was the first excitable tissue shown to exhibit a significant anion conductance (Hodgkin and Horowicz, 1959). Although the resting muscle chloride conductance largely exceeds the resting potassium conductance, chloride ions do not contribute to the resting membrane potential under physiological conditions. Variations of external [Cl$^-$] cause only a transient change in membrane potential that results in rapid redistribution of anions and return of the transmembrane voltage to the initial value (Hodgkin and Horowicz, 1959). The physiological role of the muscle chloride conductance was therefore initially unclear.

A genetic human disease, myotonia congenita, helped to clarify the impact of muscle chloride channels and provided an example of how background conductances can contribute to the modulation of cell excitability. Patients with myotonia suffer from muscle stiffness that is usually experienced as trouble releasing the grip on objects or as difficulties rising from a sitting position. An animal model of myotonia congenita, the myotonic goat, allowed detailed investigation of the underlying disease mechanisms (Bryant, 1976). Work on muscle preparations of this animal revealed that myotonia congenita is caused by an overexcitability of the muscle fiber. Myotonic muscle fibers exhibit a decreased resting chloride conductance (Lipicky and Bryant, 1973), and normal muscle fibers can be made hyperexcitable upon block of muscle chloride channels (Bryant and Morales-Aguilera, 1971). In a landmark study on muscle excitability and myotonia, Adrian and Bryant (1974) demonstrated that the existence of transverse tubules, deep invaginations of the sarcolemma that permit the quick penetration of electrical signals to the muscle interior, makes muscle chloride channels necessary for normal muscle excitability. In normal as well as in myotonic muscle fibers, a series of action potentials results in extracellular potassium accumulation and depolarization of the T-tubules. In normal muscle, a large resting chloride conductance reduces the length constant of electrotonic signal propagation and prevents transmission of T-tubular depolarization to the surface membrane. In myotonic muscle, electrotonic propagation is more effective, resulting in after-depolarization after a series of action potentials and consequent generation of muscle action potentials even in the absence of stimulation (Adrian and Bryant, 1974). Dysfunction of muscle chloride channels in myotonia does not modify the shape of the action potential (Lipicky and Bryant, 1973), and so it can be concluded that these channels do not contribute to the repolarization of the muscle fiber. Rather, chloride channels increase the resting conductance of muscle fibers, preventing sarcolemmal overexcitability upon T-tubular potassium accumulation.

Linking muscle chloride channels to one of the first known ion channelopathies and the identification of a role of anion channels in cellular excitability raised the interest of the ion channel community on this class of channels. However, for many years, muscle chloride channels resisted a detailed biophysical characterization. This changed in 1991, as the muscle chloride channel, ClC-1, was cloned (Steinmeyer et al., 1991). Heterologous expression on channels permitted detailed functional investigation of this channel. ClC-1 channels have low conductance with pronounced inward rectification (Steinmeyer et al., 1991; Pusch et al., 1994; Fahlke et al., 1995) and are perfectly suited to generate large resting chloride conductances while minimally interfering with the upstroke of the action potential. Although its physiological role is to provide a resting "leak" conductance, ClC-1 channels show exquisite voltage-dependent gating (Fahlke et al., 1996; Rychkov et al., 1996). The importance of channel gating was illustrated by naturally occurring mutations that were found in patients with myotonia congenita and

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shown to specifically affect ClC-1 gating (Pusch et al., 1995; Fahlke et al., 1995).

ClC-1 is member of the ClC family of anion channels and transporters, a large family of anion transport proteins with many unique properties (Jentsch, 2008), and studies on ClC-1 contributed significantly to our understanding of this class of transport proteins. ClC channels are voltage gated but do not contain an endogenous voltage sensor. The voltage dependence of gating transitions stems from voltage-dependent translocation of anions and protons (Lisal and Maduke, 2008). ClC channels have two protopores that function, for the main part, independently of each other (Feng et al., 2010). A large carboxy terminus that is in close contact with the ion conduction pathway (Feng et al., 2010) modifies pore properties (Hebeisen and Fahlke, 2005). The most surprising finding has been that the majority of ClC isoforms are not anion channels at all, but rather anion–proton exchangers (Accardi and Miller, 2004). Indeed, just as we had accepted that transporters outnumber channels in a family of anion channels, it became clear that coupled ClC transporters can assume channel-like slippage modes (Alekov and Fahlke, 2009), and that ClC-mediated anion–proton exchange might occur by a pore-mediated transport process (Feng et al., 2010).

In these exciting times of ClC research, one could have almost forgotten that there are physiologically important ClC channels and that these are still far from being fully understood. For many years, the prototypic mammalian ClC channel, ClC-1, could not be reliably studied in native tissue. Unitary current amplitudes are too small to permit direct observation of single-channel currents (Pusch et al., 1994), and macroscopic recordings suffered from difficulties in separating chloride currents from other current components. Fahlke and Rüdel (1995) used the Vaseline-gap technique on manually dissected fibers from rat psoas muscle. This approach enabled basic characterization of mammalian muscle chloride channels; however, the complexity in preparing healthy fibers and obtaining Vaseline seals tight enough to record current amplitudes without leakage subtraction prevented a widespread use of this technique. Moreover, the distribution of chloride channels between T-tubular membrane and sarcolemma was not known, and the quality of voltage clamp in T-tubules could not be assessed or adjusted for in theoretical analysis.

In this issue, DiFranco et al. provide a detailed analysis of ClC-1 channels in mammalian skeletal muscle fibers, with a novel and convincing method. The authors perform voltage clamp experiments on enzymatically prepared short mammalian muscle fibers using classical two-microelectrode voltage clamp. Internal anion concentrations were adjusted by pipette solutions and by clamping the muscle fiber to the respective anion reversal potential. Background currents were blocked with internal TEA and subtracted by measuring currents in the presence and in the absence of a high affinity ClC-1 blocker, 9-anthracene carboxylic acid (Bryant and Morales-Aguilera, 1971). These maneuvers resulted in time- and voltage-dependent currents that resemble ClC-1 currents in heterologous expression systems (Fahlke et al., 1996; Rychkov et al., 1996), though with slower kinetics and slight alterations in voltage dependence. Because of the short electrotonic length constants and the complicated architecture, accurate voltage clamp of muscle fibers represents a challenge. The Vergara group therefore combined electrophysiological recordings with measurements of membrane potentials using a voltage-sensitive dye, di-8-ANEPPS. These experiments demonstrated that membrane charging is much slower upon stepping to negative than to positive potentials (Fig. 3 in DiFranco et al., 2010). Moreover, block of the muscle chloride conductance resulted in a marked decrease of the membrane charging times. This dependence of voltage attenuation on the amplitude and the time course of chloride currents cannot be explained by imperfect voltage clamp, but rather indicate the presence of chloride channels in membrane compartments with large access resistance, i.e., in the T-tubules. The authors then used a radial cable model (DiFranco et al., 2007) to quantify the distribution of muscle chloride channels between sarcolemma and T-tubule, leading them to conclude that there is a homogeneous distribution of ClC-1 in these two compartments. Because the total T-tubular membrane area substantially exceeds that of the sarcolemma, the majority of ClC-1 is therefore located in T-tubular membranes. The analysis also allows for the calculation of chloride currents through the muscle sarcolemma (Fig. 4 in DiFranco et al., 2010), and these deduced currents closely resembled ClC-1 currents in heterologous expression systems.

These are beautiful experiments with a convincing analysis and a satisfying conclusion. Having read only this paper, one would be convinced that the old question about the localization of muscle chloride channels has been finally settled. However, in another publication of *The Journal of General Physiology*, Lueck et al. (2010) report experimental evidence for another subcellular distribution of ClC-1. In this paper, a combination of electrophysiology, immunofluorescence and confocal imaging, and formamide-induced detubulation is described that supports the exclusive occurrence of ClC-1 in the sarcolemma. One of the most striking findings is the comparison of electrical properties of individual muscle fibers before and after detubulation that reveals large changes in capacitance but identical chloride current amplitudes and kinetics.

Both studies are carefully executed, and obvious counterarguments have already been addressed by the authors. In the paper by Lueck et al. (2010), masking or truncating the carboxy-terminal epitope might have

produced a population of T-tubular ClC-1 undetectable in immunofluorescence experiments. However, the authors demonstrate that heterologously expressed and fluorescently tagged ClC-1 also results in exclusive staining of the sarcolemma. If ClC-1 were not present in the T-tubules, the observed chloride conductances in the T-tubules might be mediated by non–ClC-1 channels. However, DiFranco et al. (2010) show that the pharmacological profile as well as the permeation and gating properties of these channels closely resemble heterologously expressed ClC-1. Moreover, lack of ClC-1 is known to result in negligible macroscopic chloride conductance in myotonic mice (Jentsch, 2008).

This controversy is not easy to resolve. Chloride channels in T-tubules as well as in the sarcolemma can electrically stabilize muscle fibers. Novel experiments are obviously necessary. However, the quality of the presented data and the number of techniques already used make it difficult to come up with novel experiments or analyses that may resolve the controversy. Myotonia congenita has be crucial in understanding the physiology of muscle chloride channels, and maybe studying pathological conditions might again help to identify the membrane compartments these channels are residing in. Although myotonia congenita is a rare disease, the number of distinct disease-causing mutations is extensive. Some of these mutations cause little or no changes in heterologous expression systems (Hebeisen and Fahlke, 2005). Could these mutations result in hyperexcitability by altering the distribution among sarcolemma and T-tubules? Moreover, there are myotonia-causing mutations with dramatic effects on ClC-1 gating (Pusch et al., 1994; Fahlke et al., 1995) that might serve as additional tests for the contribution of T-tubular chloride conductances to macroscopic currents and transmembrane voltages. Extending the experiments by DiFranco et al. (2010) and Lueck et al. (2010) to pathological mutations in ClC-1 will possibly further improve our understanding of muscle chloride channels and muscle excitability. No matter what the final resolution will be, muscle chloride channels have once again illustrated the challenge, the complexity, and the importance of modern cell physiology.

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