

## CIC-1 chloride channel - matching its properties to a role in skeletal muscle

Edoardo C. Aromataris and Grigori Y. Rychkov

School of Molecular and Biomedical Science, University of Adelaide,  
Adelaide, South Australia 5005, Australia.

### Summary

1. CIC-1 is a  $\text{Cl}^-$  channel in mammalian skeletal muscle, which plays an important role in membrane repolarisation following muscular contraction. Reduction of CIC-1 conductance results in myotonia – a state characterised by muscle hyperexcitability.

2. CIC-1, as the other members of CIC family, exists as a dimer which forms a double-barrelled channel. Each barrel, or pore, of CIC-1 is gated by its own gate ('fast' or 'single pore' gate), while both pores are gated simultaneously by another mechanism ('slow' or 'common' gate).

3. Comparison of the biophysical and pharmacological properties of heterologously expressed CIC-1 with the properties of the  $\text{Cl}^-$  conductance measured in the skeletal muscle strongly suggests that CIC-1 is the major  $\text{Cl}^-$  channel responsible for muscle repolarisation. However, not all results obtained in the experiments on the whole muscle or muscle fibres support this notion.

4. In this review we attempt to bring together the current knowledge of CIC-1 with the physiology of skeletal muscle.

### Introduction

CIC-1 is a mammalian membrane protein of approximately 990 amino acids in length almost exclusively expressed in skeletal muscle, where it forms a  $\text{Cl}^-$  channel.<sup>1</sup> Expression of CIC-1 in skeletal muscle appears to be dependent on the extent of innervation and muscle electrical activity during development.<sup>2,3</sup> Immunocytochemistry has shown it to be specifically localised in the sarcolemmal membrane.<sup>4,5</sup> The physiological role of CIC-1 is illustrated by the pathophysiology of human inherited disease *myotonia congenita*, which results from naturally occurring mutations in the gene encoding CIC-1 (*CLCN1*). Myotonia, which can be defined as a hyperexcitability of the skeletal muscle membrane, is characterised by repetitive firing of action potentials and prolonged muscle contraction or muscle stiffness, and is a result of a reduced  $\text{Cl}^-$  conductance due to CIC-1.<sup>6,7</sup> Although comparison of the properties of the  $\text{Cl}^-$  conductance measured in skeletal muscle with the properties of heterologously expressed CIC-1 leaves little doubt that CIC-1 contributes the majority

of resting membrane  $\text{Cl}^-$  conductance in mammalian muscle, ensuring its electrical stability, some controversy still remains. In particular, physiological experiments on skeletal muscle localise  $\text{Cl}^-$  conductance to the T-tubular system.<sup>8,9</sup> Moreover, dependence of the muscle  $\text{Cl}^-$  conductance on pH cannot be explained by the pH dependence of CIC-1.<sup>10</sup>

In this short review, we focus on the evidence that supports the notion that CIC-1 is the major  $\text{Cl}^-$  channel that regulates muscle excitability and discuss possible reasons for some discrepancy between results obtained in whole muscle and heterologous expression systems.

### The role of $\text{Cl}^-$ conductance in skeletal muscle

Compared to other excitable cells, skeletal muscle has unusually high  $\text{Cl}^-$  conductance ( $G_{\text{Cl}}$ ), accounting for up to 85% of all membrane conductance at rest.<sup>11,12</sup> The large resting  $G_{\text{Cl}}$  helps to stabilise the membrane potential and contributes to the repolarisation current which terminates the action potential in skeletal muscle. Although the effect on repolarisation attributable to  $\text{Cl}^-$  channels is probably small in comparison to the action of voltage-gated  $\text{K}^+$  channels, it is nonetheless significant. To ensure that depolarisation of the sarcolemmal membrane achieves its desired effect, the T-tubules bring the depolarisation wave into close proximity with the internal  $\text{Ca}^{2+}$  stores to stimulate  $\text{Ca}^{2+}$  release. Due to the long diffusion distances within the T-tubule system of skeletal muscle, local ion concentrations cannot rapidly equilibrate with those of the bulk extracellular fluid. As a consequence, the repolarising  $\text{K}^+$  currents from trains of action potentials would increase the normally low intratubular  $\text{K}^+$  concentration after repeated action potentials, thereby shifting the  $\text{K}^+$  equilibrium potential and depolarising the membrane. One would expect this to lead to aberrant generation of new action potentials. Fortunately, the high  $\text{Cl}^-$  conductance in skeletal muscle helps to alleviate this problem.<sup>13-16</sup> As the concentration of  $\text{Cl}^-$  within the extracellular fluid is more than an order of magnitude greater than that of  $\text{K}^+$ , the effect of any  $\text{Cl}^-$  movement out of the extracellular space into the cell on the total  $\text{Cl}^-$  concentration in the extracellular fluid and the  $\text{Cl}^-$  equilibrium potential is negligible, and the  $\text{Cl}^-$  conductance is able to negate the depolarising effect of  $\text{K}^+$  accumulation.<sup>14,15</sup>

Inhibition of  $\text{Cl}^-$  conductance by  $\text{Cl}^-$  channels

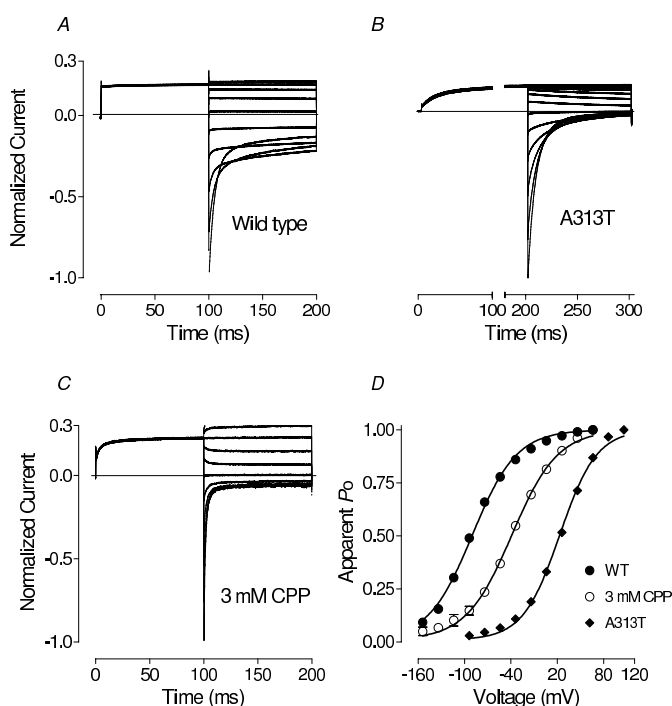
inhibitors such as anthracene-9-carboxylic acid (A-9-C) or 2-(4-chlorophenoxy)propionic acid (CPP) in muscle fibres results in myotonia, a condition in which a single action potential at a neuro-muscular junction causes repetitive action potentials on the muscle membrane and delayed muscle relaxation.<sup>6,12,17-20</sup> Similar conditions characterised by repetitive firing of action potentials and prolonged muscle contraction or muscle stiffness occur naturally due to mutations in the gene encoding CIC-1 (*CLCN1*) muscle chloride channel, causing reduction of muscle Cl<sup>-</sup> conductance. In humans and animals mutations in CIC-1 may cause following symptoms: muscle stiffness, muscle weakness, percussion myotonia – myotonic muscles become indented for many seconds following a blow with a percussion hammer, warm-up phenomena – where symptoms are alleviated after an initial period of movement, temperature dependence – symptoms are sometimes aggravated by the cold, and myotonic runs on electromyographic examination – the repetitive muscular activity which is most commonly used to diagnose the disease.<sup>21,22</sup> Muscle biopsies from myotonic goats, and subsequently humans suffering similar symptoms, first implicated a reduced G<sub>Cl</sub> as responsible for the muscular disorder.<sup>6,23,24</sup> Human myotonia related to Cl<sup>-</sup> channel dysfunction can be inherited in an autosomal dominant (Thomsen's disease) or recessive (Becker's disease) form. Of the more than 80 nonsense and missense mutations that have been identified in human patients, many more are recessive than dominant (for review see Pusch, 2002<sup>7</sup>). Truncating mutations, which are always associated with the more severe Becker type myotonia, are more commonly located in the C-terminal tail of the protein.<sup>15</sup>

### Properties of heterologously expressed CIC-1 muscle chloride channels

The recent X-ray crystallography of prokaryotic CIC proteins confirmed the idea developed from earlier electrophysiological studies that CIC channels have two identical pores.<sup>25</sup> It has been established that the transmembrane domain of CIC channels is formed by two separate subunits each containing a pore, while two interacting carboxyl tails form a cytoplasmic domain of the channel.<sup>26,27</sup> Single channel recordings of CIC-0, and later of CIC-1 suggested that these channels have two independent gating processes: 'fast gating' or 'single pore gating' which acts on each individual pore of the double-barrelled channel, and 'slow gating' or 'common gating' which acts on both pores simultaneously.<sup>28-30</sup>

When expressed in heterologous systems, CIC-1 shows voltage-dependent Cl<sup>-</sup> currents that deactivate at hyperpolarizing potentials and saturate at depolarising potentials (Figure 1).<sup>31-34</sup> Gating of CIC-1 is both voltage and Cl<sup>-</sup> dependent similar to the fast gating of a homologous Cl<sup>-</sup> channel, CIC-0, expressed in *Torpedo* electric organ.<sup>35</sup> Cl<sup>-</sup> dependence of both channels is evident when extracellular Cl<sup>-</sup> concentration is changed. When extracellular Cl<sup>-</sup> concentration is reduced 10 fold, the open probability curve of the fast gate of CIC-0 is shifted by

~40 mV towards hyperpolarizing potentials, while the open probability curve of CIC-1 is shifted by ~58 mV.<sup>34-37</sup> Investigation of CIC-0 single channel kinetics at different Cl<sup>-</sup> concentrations led to a model of CIC-0 fast gating in which voltage independent Cl<sup>-</sup> binding to an external binding site is followed by a transfer of the bound Cl<sup>-</sup> across the electric field during the conformational change of the channel protein.<sup>35,36</sup> In such a model, voltage-dependence of the fast gate arises from interactions with the permeating anion rather than through an intrinsic voltage-sensing mechanism. A similar model was later adopted for CIC-1.<sup>38</sup>



**Figure 1. Effect of dominant myotonic mutation (A313T) and 2-(4-chlorophenoxy) propionic acid (CPP) on CIC-1 gating.** CIC-1 currents recorded from HEK293 cells expressing the WT human CIC-1 channel (A), A313T mutant (B) and in the presence of 3mM CPP in the bath solution (C). In A and C, prepulse to +40 mV was followed by voltage steps ranging from -120 mV to +80 mV in 20 mV increments. In B, prepulse to +120 mV was followed by voltage steps ranging from -120 mV to +120 mV in 20 mV increments. Holding potential was -30 mV in all cases. Currents are normalized to the peak current amplitude recorded at -120 mV. The effect of both the A313T mutation and 3 mM CPP on CIC-1 open probability ( $P_o$ ) is shown in D ( $n = 6 - 9$ ). Apparent  $P_o$  was estimated from the peak tail currents after the voltage steps to different membrane potentials. Solid lines represent the Boltzmann distribution.

The structural basis of fast gating has been deduced from the crystal structure of the prokaryotic members of CIC family. Although these proteins are not channels, but transporters, it has been suggested that a side chain of

glutamate, E148, may correspond to the fast gate of CIC channels.<sup>25,27,39</sup> This amino acid residue is highly conserved through members of the CIC family and corresponds to E232 in hCIC-1. From analysis of the crystal structure, it appears that for Cl<sup>-</sup> conduction to occur, this gate must swing out of the way. Presumably, this occurs when a Cl<sup>-</sup> ion from the extracellular solution enters the pore and displaces the glutamate side chain due to charge interactions.<sup>25,27,40</sup>

The common gating of CIC channels, including CIC-1, is much less understood. No structure has yet been identified that could represent the common gate. It is clear, however, that common gating involves significant structural re-arrangements of the protein, as temperature dependence of this type of gating is unusually high.<sup>41-43</sup> It is also clear that it involves interactions between two subunits and possibly between the carboxyl tails. Mutations that affect common gating have been found in different parts of the protein, including the carboxyl tail, but there is clustering of such mutations on the interface between the subunits where the interaction is likely to occur.<sup>43</sup> Nearly all mutations that result in dominant type myotonia congenita shift the voltage dependence of CIC-1 common gating to more positive potentials.<sup>30,43-50</sup> The shift of CIC-1 activation to positive potentials reduces Cl<sup>-</sup> currents within the physiological range of voltages, resulting in the muscle hyperexcitability characteristic of myotonic disease (Figure 1). Most of the dominant negative mutations also change the CIC-1 kinetics, slowing down current activation at depolarising potentials, which contributes to the reduction of the Cl<sup>-</sup> conductance in myotonic muscle.<sup>43</sup> The dominant negative effect of these mutations is illustrated in experiments where mutant subunits impose their altered gating on mutant/wild type heterodimers.<sup>30,44-46,51</sup>

The fact that dominant negative mutations that shift  $P_o$  of the slow gating to more positive potentials can cause myotonia provides strongest evidence that of all types of Cl<sup>-</sup> channels present in the skeletal muscle, only CIC-1 is responsible for regulation of its excitability. With only one allele carrying dominant mutation there is a 25% chance that both monomers comprising a CIC-1 dimer are wild type. Therefore, individuals that have dominant myotonia congenita must have skeletal muscle Cl<sup>-</sup> conductance of at least 25% of the norm, due to the presence of wild type/wild type CIC-1 dimers. On the other hand, to induce myotonic runs in muscle, pharmacological experiments have shown that muscle  $G_{Cl}$  should be reduced by around 75%,<sup>52,53</sup> suggesting a large safety margin prior to any noticeable symptoms of the disease. In the presence of 25% of normal CIC-1 conductance any significant additional Cl<sup>-</sup> conductance due to a presence of other Cl<sup>-</sup> channels would render myotonic muscle normal. Therefore, taken together these results indicate that the role of other than CIC-1 Cl<sup>-</sup> channels in regulation of skeletal muscle excitability must be negligible.

The comparison of the biophysical and pharmacological properties of the heterologously expressed CIC-1 with the properties of skeletal muscle Cl<sup>-</sup> conductance strongly supports this notion. Currents

through CIC-1 expressed in Sf-9 insect cells and HEK293 cells inactivate at negative potentials in agreement with macroscopic Cl<sup>-</sup> currents recorded in muscle cells.<sup>54,55</sup> At pH 5.5 and below the voltage dependence of CIC-1 is reversed and voltage steps to negative potentials elicit activating currents.<sup>34,42</sup> Similar transition of normally inactivating Cl<sup>-</sup> currents at pH 7 into activating currents at pH 5 has been shown in muscle fibres.<sup>11</sup> The complete permeability sequence of heterologously expressed CIC-1 is SCN<sup>-</sup> > ClO<sub>4</sub><sup>-</sup> > Cl<sup>-</sup> > Br<sup>-</sup> > NO<sub>3</sub><sup>-</sup> > ClO<sub>3</sub><sup>-</sup> > I<sup>-</sup> > BrO<sub>3</sub><sup>-</sup> > HCO<sub>3</sub><sup>-</sup> > F<sup>-</sup>, while just for halides it is Cl<sup>-</sup> > Br<sup>-</sup> > I<sup>-</sup> > F<sup>-</sup>.<sup>56</sup> This sequence corresponds to a cationic site of moderately strong field strength in the membrane (Eisenman sequence 4).<sup>57</sup> From blocking ability when applied externally, the binding sequence is SCN<sup>-</sup> > ClO<sub>4</sub><sup>-</sup> > I<sup>-</sup> > NO<sub>3</sub><sup>-</sup> > Br<sup>-</sup> > BrO<sub>3</sub><sup>-</sup> > HCO<sub>3</sub><sup>-</sup> > F<sup>-</sup>, which corresponds to the low field strength Eisenman sequence 1.<sup>57</sup> These results agree with early studies of foreign anion permeability and of block of <sup>36</sup>Cl<sup>-</sup> efflux by foreign anions in frog muscle where the same sequence difference occurred.<sup>58,59</sup> The stereospecific actions of clofibric acid derivatives, in particular 2(4-chlorophenoxy) propionic acid, or CPP, also point to CIC-1 being the major skeletal muscle chloride channel (Figure 1). When applied to mammalian muscle, the decrease in  $G_{Cl}$  and myotonic symptoms observed are entirely attributable to the S(-) enantiomer of the compound.<sup>20,60</sup> Similarly, when applied to heterologously expressed rat and human CIC-1 channels, a stereospecific reduction of channel open probability was discovered.<sup>7,37,49,61</sup> This mechanism of action of CPP and its derivatives is similar to the mechanism by which naturally occurring dominant mutations in *CICN1* result in myotonic symptoms in muscle.<sup>49</sup>

### CIC-1 localisation in skeletal muscle

Despite overwhelming evidence that CIC-1 is the major Cl<sup>-</sup> channel in the skeletal muscle some discrepancies still remain. In the first instance, it is currently unresolved whether the high Cl<sup>-</sup> conductance of mammalian skeletal muscle resides in the sarcolemma or in the T-tubules. With repetitive muscle stimulation K<sup>+</sup> accumulates more in the narrow T-tubular system than in the extracellular space, so one would expect to find at least some Cl<sup>-</sup> conductance in the T-tubular membrane. Recent immunohistochemical evidence suggested that CIC-1 is particularly concentrated in the sarcolemmal membrane rather than the T-tubule system.<sup>4,5</sup> These results differ dramatically from previous physiological localisation of skeletal muscle Cl<sup>-</sup> channels. Using glycerol treatment,  $G_{Cl}$  in mammalian muscle was previously shown to be similar on surface and T-tubular membranes which when corrected for surface area suggested that up to 80% of Cl<sup>-</sup> conductance may be associated with T-tubules.<sup>8,11,62</sup> Moreover, the presence of a large Cl<sup>-</sup> conductance in the T-tubular system, which is up to 4 times larger than the K<sup>+</sup> conductance, has been shown in mechanically skinned rat fibres with the sarcolemmal membrane removed.<sup>9</sup> As CIC-1 does not appear to be present in T-tubules using both

immunofluorescence and cell fractionation techniques, it has been suggested that a distinctly different  $\text{Cl}^-$  channel may be present in T-tubules.<sup>4,9</sup> Several types of single  $\text{Cl}^-$  channel currents have been recorded from sarcolemma and T-tubular membrane, but there is no evidence that they contribute to macroscopic  $\text{Cl}^-$  conductance recorded from skeletal muscle<sup>63,64</sup> and, as noted earlier, it is CIC-1 that is important in preventing myotonic contractions.

One possible explanation for these conflicting results may come from a recent study that shows redistribution of CIC-1 during processing of skeletal muscle. While immunolocalisation studies of the CIC-1 in rat skeletal muscle cryosections showed a prominent sarcolemmal staining, in single myofibres cultured in Matrigel all of CIC-1 specific staining is intracellular. The lack of sarcolemmal CIC-1 was observed immediately after the isolation of the fibres. Protein kinase inhibitors and electric stimulation of the fibres returned the CIC-1 to the sarcolemma.<sup>5</sup> This suggests that localisation of CIC-1 in the muscle is strongly regulated and that CIC-1 rapidly internalises when muscle fibres are isolated and maintained in cell culture conditions. These results could offer several explanations for discrepancy between physiological and immunological localisation of CIC-1. It is possible that glycerol treatment, which is used to seal the T-tubular system, also disrupts sarcolemmal  $\text{Cl}^-$  conductance by facilitating CIC-1 internalisation, while in skinned fibres, in the absence of sarcolemma, the CIC-1 internalised during the isolation procedure is incorporated in T-tubular membrane after the fibre has been electrically stimulated. Another possibility is that CIC-1 is, in fact, present in the T-tubules of intact skeletal muscle as well as in the sarcolemma but is not visible with the antibody that has been used. This antibody binds to residues in the carboxyl tail of CIC-1, and it is possible that when CIC-1 is localised in T-tubules this part of the carboxyl tail of CIC-1 interacts with the intracellular proteins rendering CIC-1 unavailable for antibody binding.

### Muscle excitability and dependence of CIC-1 on pH

Another point of contention is the pH dependence of muscle excitability and pH dependence of heterologously expressed CIC-1. Experiments on skinned muscle fibres and isolated rat soleus muscles suggested that recovery of excitability in  $\text{K}^+$ -depressed muscles induced by acidification is related to reduction in the inhibitory  $\text{Cl}^-$  conductance.<sup>10,65</sup> It has been shown that lowering pH from 7.4 to 6.8 causes a significant reduction (~46%) of the muscle  $\text{Cl}^-$  conductance and a concurrent recovery of action potentials and force in muscle in the presence of high extracellular  $\text{K}^+$  (9-11 mM).<sup>10</sup> Similar recovery of force and excitability could be achieved at normal pH by blocking  $\text{Cl}^-$  conductance by A-9-C. The authors concluded that short-term regulation of  $\text{Cl}^-$  channels is important for maintenance of excitability of working muscle. These results suggested that CIC-1 expressed in the muscle is strongly pH dependent in a narrow range of pH between 7.4 and 6.8.

The pH dependence of CIC-1 expressed heterologously in Sf-9 and HEK 293 cells has been well characterised.<sup>42,66</sup> When pH is lowered to 5.5 and below the voltage dependence of CIC-1 is reversed and at this pH CIC-1 is opened by hyperpolarization and is inactivated at depolarised potentials. As a consequence, the outward current through CIC-1, which in the muscle is responsible for membrane repolarization, is smaller at pH 5.5 than at normal pH.<sup>42</sup> In addition, the hyperpolarization-activated gating of CIC channels, including CIC-1, strongly depends on intracellular  $\text{Cl}^-$  concentration, with open probability dropping to nearly zero at muscle resting potential at 4 mM internal  $\text{Cl}^-$ ,<sup>67</sup> which might explain low  $\text{Cl}^-$  conductance in the muscle at pH below 5.5. However, when the pH is lowered from 7.4 to 6.8, there is only a small increase of open probability of heterologously expressed CIC-1 at negative potentials,<sup>34</sup> which does not explain the decrease of  $\text{Cl}^-$  conductance seen in the muscle. It is known that gating of CIC-1 is altered by foreign anions present either extra- or intracellularly.<sup>56</sup> Most of them, including some organic anions, block CIC-1.<sup>56,68</sup> It is also known that block of CIC-1 by the anions of carboxylic acids is strongly pH dependent.<sup>37,66</sup> There is a range of endogenous organic anions present in the muscle, which might block CIC-1 or effect its gating. It is possible that even moderate acidification of the muscle is sufficient to substantially increase the block of CIC-1 by one of these anions, which would result in reduction of muscle  $\text{Cl}^-$  conductance.

The possibility that regulation of CIC-1 activity in muscle might be more complicated than previously thought is well demonstrated by recent finding that CIC-1 gating is modulated by intracellular nucleotides.<sup>69</sup> Addition of 5 mM ATP to the intracellular solution shifts open probability of the common gate of heterologously expressed CIC-1 by ~50 mV towards depolarising potentials, essentially reducing  $\text{Cl}^-$  conductance at muscle resting potential ~6 fold. Other adenosine nucleotides, ADT and AMP, have a similar effect. The product of AMP deamination, IMP, however, has no effect on CIC-1 open probability. In fast twitch muscles during intense sprint exercise ATP can be depleted to levels below 1 mM and accumulated as IMP.<sup>70</sup> This opens a possibility that under conditions of sprint exercise or ischemia, open probability of CIC-1 might increase due to the fall in ATP levels, thus reducing muscle excitability and contributing to fatigue. It is tempting to speculate that such a mechanism of CIC-1 regulation by ATP would protect cells both from the toxic effects of prolonged depolarisation and, by reducing ATP demand, from complete ATP depletion and  $\text{Ca}^{2+}$ -dependent damage. However, there is no evidence so far that dependence of CIC-1 on ATP has any role in regulation of muscle excitability, and the only indication that such mechanism might exist comes from the early experiments conducted on metabolically exhausted muscle fibres showing about a 15 fold increase in  $\text{Cl}^-$  conductance.<sup>71</sup>

Another recently discovered property of CIC-1 that could be relevant for muscle physiology is the regulation of pH. CIC-1, apart from being a  $\text{Cl}^-$  channel, is also a  $\text{Cl}^-/\text{H}^+$  antiporter.<sup>72</sup> Similar to gating of some other members of the

ClC family, gating of ClC-1 involves transport of protons across the membrane.<sup>72,73</sup> This movement of protons through ClC-1 over expressed in *Xenopus* oocytes is enough to change the pH by up to 0.2 units on either side of the membrane, depending on the membrane potential. Yet again, it is not known whether this property of ClC-1 has any physiological role in muscle.

### Conclusions

Since ClC-1 has been cloned and studied in heterologous expression systems, it has become apparent that it is regulated by the extracellular and intracellular anion binding sites, extracellular and intracellular pH, and intracellular adenosine nucleotides. It has been shown that the expression of ClC-1 on the sarcolemma is tightly regulated by phosphorylation and depends on electrical activity of the muscle. Taken together these results suggest that intracellular ATP, anionic metabolites, and pH may use ClC-1 as one of the pathways that regulate the excitability of working muscle. The extent to which ClC-1 is involved in regulation of muscle excitability according to physiological conditions might not be fully appreciated and requires further investigation.

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*Author for correspondence:*

Dr. Grigori Rychkov  
School of Molecular and Biomedical Science  
University of Adelaide,  
Adelaide, South Australia, 5005, Australia.

Tel: +61 8 8303 3979

Fax: +61 8 8303 3356

Email: Grigori.Rychkov@adelaide.edu.au