Response to the letter: "On the localization of ClC-1 in skeletal muscle fibers"

John D. Lueck,[1,3,6] Ann E. Rossi,[1] Charles A. Thornton,[2] Kevin P. Campbell,[3,4,5,6] and Robert T. Dirksen[1]

[1]Department of Pharmacology and Physiology, and [2]Department of Neurology, University of Rochester, Rochester, NY 14642
[3]Department of Molecular Physiology and Biophysics, [4]Department of Internal Medicine, [5]Department of Neurology, and [6]Howard Hughes Medical Institute, Roy J. and Lucille A. Carver College of Medicine, University of Iowa, Iowa City, IA 52242

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We are writing in response to the Letter to the Editor by Lamb et al. in this issue, which challenges the central conclusion of our recent study published in *The Journal of General Physiology*, namely that "functional ClC-1 channels in adult mouse skeletal muscle reside exclusively within the sarcolemma" (Lueck et al., 2010).

The primary source of concern expressed by Lamb et al. relates to the acquisition and interpretation of our confocal images. The first issue raised is that endogenous ClC-1 channel immunoreactivity may have been missed in the X–Y confocal images because of the small T-tubule diameter, limited pixel size, and insufficient photomultiplier tube gain. However, Lamb et al. fail to acknowledge that our confocal imaging was able to clearly resolve T-tubule di-8-ANNEPS (Fig. 1 A) and expressed GFP-α bungarotoxin–labeled ΔRamp (Fig. S1 A, BTX-ΔRamp) fluorescence, conditions expected to produce comparable labeling of both the sarcolemmal and T-tubule membranes. Although the T-tubule signals for these probes were indeed lower than that of the sarcolemma, as expected, a prominent T-tubule signal was clearly resolved with both fluorophores. Importantly, similar results were observed for Cav-3, which localizes to both the sarcolemmal and T-tubule membranes (Ralston and Ploug, 1999; Murphy et al., 2009). Thus, we would have easily observed a T-tubule ClC-1 fluorescence if the native ClC-1 T-tubule density was significant or at least comparable to that within the sarcolemma.

Additional concerns were raised regarding the ability of formamide to detubulate skeletal muscle based on an apparent reduction in sarcolemmal di-8-ANEPPS fluorescence after detubulation in the representative image shown in Fig. 1 B of Lueck et al. (2010). Because the two images shown in this figure come from different fibers stained with di-8-ANNEPS at different times, inferences based on a direct comparison of the sarcolemmal signals between the two images cannot be made. However, differences between the ratio of the T-tubule and sarcolemmal signals in each image do provide an index of the effect of detubulation on dye staining of the two membrane compartments. For the images shown in Fig. 1 (A and B), the ratio of T-tubule to sarcolemmal signal was 0.27 (23.4/86.4) in control and ∼0 (0.08/71.1) after detubulation. In addition, membrane potentiometric dyes have been used by others and demonstrated to be a valid means of assessing T-tubule uncoupling and disorganization (He et al., 2001; Brette et al., 2002). Most importantly, the reduction in T-tubule di-8-ANEPPS staining as an indicator of successful detubulation in our study was directly substantiated by confirmation of reduced membrane capacitance in whole cell patch clamp experiments after formamide-induced osmotic shock (Figs. 4 B and 6 D).

Lamb et al. also expressed concerns regarding our localization of exogenously expressed GFP-ClC-1 channels in mouse skeletal muscle (Fig. 10). We agree that unlike native ClC-1 localization discussed above, significant internal GFP-ClC-1 fluorescence is observed after adenoviral-mediated GFP-ClC-1 expression in our experiments. Indeed, images of some Ad-GFP-ClC-1–infected fibers showed significant intracellular fluorescence organized in circular rings, consistent with an association with T-tubules (Fig. 10 A), whereas other fibers showed a more global increase in fluorescence that is not consistent with T-tubule localization (Fig. 10 D). A clear limitation of such adenoviral-mediated expression experiments is that the protein may be overexpressed, resulting in protein aggregation along the synthesis, trafficking, and degradation pathways. The possibility that intracellular fluorescence in Ad-GFP-ClC-1–infected fibers is not properly inserted in the T-tubule membrane is highlighted by the fact that only sarcolemma-associated ClC-1 channels containing an extracellular α-BTX–binding site were labeled with Alexa 594–conjugated α-BTX, whereas clear T-tubule labeling was observed after expression of a control α-BTX–labeled membrane protein (α-BTX-ΔRAMP1) (Fig. S1 F). Nevertheless, we cannot conclude with any certainty that T-tubule ClC-1 expression does not occur

Correspondence to John D. Lueck: john-lueck@uiowa.edu

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in some fibers under conditions after infection with Ad-GFP-ClC-1. Indeed, as pointed out by Lamb et al., DiFranco et al. (2009) demonstrated that expression of EYFP-ClC-1 in mouse FDB muscle fibers by cDNA electroporation can result in double rows of transverse EYFP-ClC-1 fluorescence, increased ClC-1 conductance, and markedly reduced excitability, as a result of successfully driving functional EYFP-ClC-1 channel expression to the surface membrane.

Lamb et al. raised the concern that a T-tubule ClC-1 conductance may have been missed in our experiments because our intracellular recording solutions lacked ATP and adequate control for the oxidative status of the cell. Although we agree that ClC-1 activity in skeletal muscle may be influenced by ATP and redox status, it is unlikely that our recording conditions would have selectively and completely silenced ClC-1 channels in the T-tubule system, but not in the sarcolemma. Lamb et al. also suggest that the use of an external solution with a higher osmolarity in the adult fiber experiments may explain why ClC-1 channel activity within the T-tubule system was not observed in our experiments. To our knowledge, the effects of osmolarity on ClC-1 activity have not been determined in mammalian skeletal muscle. Although we cannot rule out potential modulatory effects of the recording conditions used in our study on ClC-1 activity, again it is unlikely that these conditions would result in selective and complete elimination of T-tubule ClC-1 function.

An important aspect of our study is that sarcolemmal-restricted ClC-1 localization was confirmed for fibers isolated from both young and old mice and using multiple complementary approaches. The importance of proper ClC-1 activity becomes evident by 14 days of age in mice, where the loss of ClC-1 function results in severe myotonia (Heller et al., 1982). Lamb et al. also suggest, and we agree, that differences among species may account for some of the disparate conclusions in the literature regarding ClC-1 subcellular localization. They correctly point out that our experiments were conducted entirely in mouse muscle fibers, whereas previous studies of ClC-1 localization in muscle have used several other species, including frog (Hodgkin and Horowicz, 1960; Eisenberg and Gage, 1969), goat (Bryant, 1970), toad (Coonan and Lamb, 1998), and rat (Palade and Barchi, 1977; Dulhunty, 1979; Coonan and Lamb, 1998; Papponen et al., 2005; Dutka et al., 2008). Future work using multiple complementary approaches is needed to carefully compare relative ClC-1 subcellular distribution across different species.

The results of our work are in opposition to a series of elegant studies using mechanically skinned muscle fibers (mostly rat), which provides evidence of a large T-tubule chloride conductance (Coonan and Lamb, 1998; Pedersen et al., 2004; Dutka et al., 2008). Although conducted with great technical skill, we disagree with the assertion of Lamb et al. that these studies provide "quite definitive evidence" for a large T-tubule chloride conductance in rat skeletal muscle. In addition to possible species differences, acknowledged above, the skinned fiber experiments provide only indirect measures of total chloride conductance based on measurements of tension development under various ionic conditions. In addition, these measurements are conducted only after mechanical disruption of the sarcolemma, which could result in either ClC-1–containing portions of the sarcolemma being used to seal over the T-tubule opening or altered trafficking and redistribution of ClC-1 channels from the artificially disrupted sarcolemma to the intact T-tubule system. Finally, Papponen et al. (2005) found that maintenance of ClC-1 within the sarcolemma is a highly regulated process that depends on the physiological environment of the cell. This study showed that ClC-1 channels in rat muscle fibers redistribute intracellularly after isolation. Thus, it is possible that some ClC-1 channels may redistribute from the sarcolemma to the T-tubule membrane during rat fiber isolation and subsequent mechanical skinning. A major strength of the experiments conducted in our study is that ClC-1 channel function was directly assayed both before and after selective disruption of the T-tubular membrane within the same muscle fibers. In addition, our experiments extended these electrophysiological studies to fibers from both young and adult mice and confirmed the findings using additional direct measures of sarcolemmal ClC-1 protein localization (immunocytochemistry and GFP/α-BTX labeling).

We would like to point out two errors in our paper that have recently come to light. First, during final formatting of the manuscript, we mistakenly inserted Adrian and Bryant (1974) instead of Bryant (1970) in reference to the findings that membrane resistance is not increased in normal goat skeletal muscle fibers after detubulation, and that myotonia persists in fibers from myotonic goats after detubulation. Additionally, we made an unintentional display error when preparing the final version of Fig. 10 C. Although, as pointed out by Lamb et al., the gain of the green channel in this panel is slightly decreased from the original intensity shown in Fig. 10 A, the conclusions drawn from this figure remain unchanged.

In conclusion, our study was the first to combine comprehensive electrophysiological and multiple confocal imaging approaches to determine the subcellular distribution of ClC-1 channels in skeletal muscle fibers from both young and adult mice. The results of these studies are clear and entirely consistent with the conclusion that functional ClC-1 channels in adult mouse skeletal muscle reside exclusively within the sarcolemma. Although Lamb et al. raise valid questions, our answers to these questions add additional strength to this conclusion.

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