On the localization of ClC-1 in skeletal muscle fibers

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We read with interest the recent article by Lueck et al. (2010) and the related commentary by Zifarelli and Pusch (2010) and wish to challenge the conclusion reached from their published data that ClC-1 chloride channels in adult mammalian skeletal muscle fibers are located "within the sarcolemma and not the T-tubular system" (Lueck et al., 2010).

First, the confocal imaging provided cannot be used as evidence that there are many more ClC-1 channels in the sarcolemma than in the T-tubular system. This is because the average diameter of the T-tubules is severalfold smaller (<50 nm) than the effective pixel size (>200 nm, set by confocal resolution), and the T-tubules are distributed throughout the volume of the fiber, whereas the surface membrane is concentrated in the same "line" in the X–Y plane. Therefore, one would need a considerably higher gain to visualize the ClC-1 channels in the T-tubules than in the sarcolemma, even if the ClC-1 channels were equally distributed between the sarcolemma and the T-tubules. Thus, the fact that ClC-1 can be seen at the surface membrane and not deeper in the fiber in Fig. 7 A is completely expected from the low gain image used. This is precisely what we have previously vividly shown in regard to caveolin-3, a protein that is often mistakenly said to be present only at the sarcolemma and not in the T-system. This protein in fact is present in very large amounts in the T-tubular system, which is readily apparent if the confocal section is examined at adequate photomultiplier gain (e.g., Fig. 6 in Murphy et al., 2009), whereas at lower gain, only the sarcolemmal caveolin-3 is apparent (see Fig. 9 in Murphy et al., 2009) in an image looking almost identical to that in Fig. 7 A in Lueck et al. (2010). Concerning the detubulation experiments shown in Fig. 1, we also query why the surface membrane staining by di-8-ANEPPS is reduced after detubulation (A, right) and whether the gain used allows adequate visualization of the T-system, particularly if it is dilated and distorted after the detubulation procedure.

Second, Lueck et al. (2010) misinterpret their confocal images of GFP-tagged ClC-1 expression in muscle (Figs. 9 A and 10) as showing "robust sarcolemma expression and a low level of intracellular localization in some fibers." The authors evidently base this on the relatively strong intensity fluorescence signal from the sarcolemma (see point above) compared with that in the fiber. For a correct interpretation of their data, they need to compare the total fluorescence signal from the sarcolemma with the total fluorescence signal from inside of the cell. From Figs. 9 A and 10 A, it appears that the total fluorescence signal from inside the cell is greater than the total fluorescence signal of the entire sarcolemma, thus contradicting the authors' claim above and the related statement made in the accompanying commentary (Zifarelli and Pusch, 2010) that "the fluorescence signal from tagged ClC-1 was confined to the sarcolemma." Furthermore, when the transverse section in Fig. 10 A is examined closely, it appears that the intracellular ClC-1 signal is organized in "rings" ∼1 µm in diameter, as would be expected if it originates from the T-tubules wrapped around individual myofibrils, very much like that seen with caveolin-3 (see Fig. 4 B in Murphy et al., 2009). We also find it rather puzzling that the authors discard the possibility that a substantial amount of GFP-tagged ClC-1 chloride channels is expressed in the T-tubules, considering that they cite the paper by DiFranco et al. (2009), where it is clearly shown that EYFP-tagged ClC-1 is predominantly expressed at T-tubular level in mouse FDB muscle fibers.

Third, when considering the question of whether the intracellularly tagged ClC-1 chloride channels in Fig. 9 A colocalize with the RYRs, Lueck et al. (2010) show the green signal from GFP-tagged CLC-1 chloride channels in a "merged" image in Fig. 10 C at a lower intensity than when it is shown in isolation in panel A, whereas the red signal from the RYRs is saturated and is merged at an unchanged intensity level. The reduced intensity of the green signal, together with the fact that the red pixels are saturated, certainly would obscure, rather than reveal, any colocalization of the red and green signals. For proper examination of any colocalization, the green and the red signals need to be of similar intensity to allow one to determine whether the green signals fall between the red pixels or whether they colocalize, in which case the color of the respective pixels would change to yellow (note that only equal amounts of red and green make yellow in additive color mixing).

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Fourth, we question several aspects related to the electrophysiological measurements in the study by Lueck et al. (2010). For example, the intracellular solution in the voltage clamp experiments did not contain any ATP, even though this has been claimed to greatly influence the properties of ClC-1 (Bennetts et al., 2007; Zhang et al., 2008). There was also no consideration of whether such intracellular conditions affected the mitochondrial and oxidative status of the cell, as the ClC-1 function is known to be affected by oxidative status, and oxidants are known to be produced at the T-system (Hidalgo et al., 2006). The extracellular solution used in the voltage clamp experiments on the adult mouse fibers had an osmolality $\sim$80 mosmol kg^{-1} higher than the internal solution (Lueck et al., 2010), and such a disparity was previously shown to increase the rate of charging of the T-system in rat muscle fibers (Lamb, 1986), seemingly by dilating the T-tubules. Both the local generation of oxidants and the dilation of the T-system and consequent membrane stretching could be further reasons why the chloride conductance in the T-system appeared to be small in the present experiments (Lueck et al., 2010).

Finally, we note that no mention is made in the article by Lueck et al. (2010), or in the accompanying commentary by Zifarelli and Pusch (2010), about the fact that the data pointing to a tubular location of much of the ClC-1 was obtained in adult rat muscle fibers, which are larger in diameter and have a much more extensive T-tubular system than mouse muscle fibers (Palade and Barchi, 1977; Dulhunty, 1979; Coonan and Lamb, 1998; Pedersen et al., 2004; Dutka et al., 2008). This is of course a critical issue, given that problems with K$^+$ accumulation will be more marked in a larger T-system, as too would be the importance of having Cl$^-$ channels localized there rather than solely at the surface. The extensiveness of the T-system in rat muscle fibers, and the difficulty of charging it by current flow from the surface, is shown by its relatively high capacitance and slow charging, which upon imposing a voltage step takes on average $\sim$6 ms to reach 90% of its final level (e.g., Fig. 7 in Lamb, 1986) compared with less than $\sim$1.5 ms in adult mouse fibers (Fig. 6 B in Lueck et al., 2010). We also point out that Lueck et al. (2010) offer no explanation for our quite definitive evidence from "skinned" muscle fibers, where the sarcolemma has been removed, that the T-system still retains a large and functionally very important chloride conductance with all the properties of ClC-1; namely, that it is blocked by 9-anthracene carboxylic acid, by low pH, and also by activating PKC with phorbol esters (Coonan and Lamb, 1998; Pedersen et al., 2004; Dutka et al., 2008). (Note that such skinned fiber recordings can be made in <10 min after dissection from a fresh muscle, and that it only takes a matter of seconds to mechanically peel back and remove the sarcolemma.) We also note that DiFranco et al. (2011)

have recently confirmed the presence of a large chloride conductance in the T-system of adult mouse skeletal fibers. Lastly, we believe that it is somewhat misleading to make the claim that the detubulation experiments in goat muscle fibers (Adrian and Bryant, 1974) support either "the notion that the majority of ClC-1 are located in the sarcolemma" (Lueck et al., 2010) or that such experiments "indicated predominant sarcolemma localization of G_{Cl}" (Zifarelli and Pusch, 2010), when the location of the chloride conductance was not investigated in those experiments. In fact, we note that the primary author on those detubulation experiments later wrote that "it is generally agreed that chloride conductance is distributed over both the surface and T-system membranes in mammalian muscle" (Heiny et al., 1990).

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