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COMMENTARY

Measurement of intracellular ion activity in skeletal muscle fibers: Four microelectrodes or no deal

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The skeletal muscle fiber exchanges ions with its extracellular medium at rest and during activity. These ion movements mostly condition the value of the membrane potential, which itself controls the contractile state of the fiber (Hodgkin and Horowicz, 1959). In this context, the study of ion movements is crucial in skeletal muscle. One of the earliest technical approach that was used to investigate transmembrane ion movements was electrophysiology. From the use of intracellular microelectrodes and the double sucrose gap technique in frog muscle fibers (Adrian et al., 1970; Allard and Rougier, 1992) to intracellular microelectrodes in enzymatically isolated mouse muscle fibers (Ursu et al., 2005; DiFranco et al., 2011; Lefebvre et al., 2014), electrophysiology has allowed changes in membrane potential and current produced by transmembrane ion fluxes to be monitored in real time and with high accuracy during activity and at rest. In this issue, Heiny et al. describe a powerful method to measure absolute intracellular ion activity in isolated skeletal muscle fibers using a combination of four microelectrodes.

All ion fluxes in muscle are under the tight control of membrane voltage, which conditions the electrochemical force pushing the ions across the membrane. For this reason, the measurement or control of membrane potential is instrumental for investigating ion fluxes. By measuring the current produced by the net movement of a given transmembrane ion flux, it is possible to estimate the consequent change in the internal concentration of ions. However, under these conditions, the absolute value of the internal concentration cannot be determined. Moreover, the ion current of interest must be isolated by pharmacological compounds, if available, or by excluding other ion species, a maneuver that often perturbs ion permeation. Also, voltage clamp is not helpful to investigate movements of a specific ion species if ions do not flow through ion selective channels and are instead transported by nonelectrogenic carriers, symporters or antiporters, or through nonselective ion channels.

In parallel, different methods have been implemented to measure ion movements and to determine the absolute intracellular concentration of a given ion. The most widespread of these methods is the use of fluorescent indicators that bind to specific ion species (Grynkiewicz et al., 1985). In skeletal muscle, the measurement of intracellular Ca^{2+} movements is the most prevalent because Ca^{2+} is mostly exchanged between the cytosol and the sarcoplasmic reticulum, which is not accessible to microelectrodes. Fluorescent indicators have been also developed to measure intracellular concentrations of monovalent ions such as Na^+, K^+, Cl^-, or H^+, and some of them have been used in skeletal muscle (Fuster et al., 2017a,b). Recordings of relative changes in fluorescence intensity emitted by the dye can be quite easily achieved, but introduction of a controlled concentration of the dye into the cell, in-cell calibration of the indicator, and limited range of concentration changes that can be detected represent limiting factors to the use of fluorescent dyes for measuring absolute intracellular ion activity.

With the development of electrophysiological approaches and the widespread use of microelectrodes, ion-sensitive microelectrodes (ISM), in particular those using liquid membrane systems, have been also introduced in mammalian skeletal muscle for measuring Cl^- or Ca^{2+} ion activities (Lopez et al., 1986; Harris and Betz, 1987). This technique involves filling the tip of a silanized microelectrode with an organic phase enriched with an ionophore specific for the ion of interest, and backfilling the micropipette with saline containing the same ion of interest. By fixing the concentration of the ion of interest in the electrode lumen, the concentration of the ion to be measured at the tip of the electrode is revealed as the Nernstian voltage difference created between the tip and the lumen of the electrode separated by the liquid organic phase selectively permeable to the ion. When impaled into cells, the voltage

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difference read with such ISM, called single-barreled ISM, is the sum of the Nernstian voltage generated by the ion gradient plus the cell membrane potential that must be simultaneously measured and subtracted. This measurement of membrane potential can be performed with another microelectrode or by using a double barreled ISM that combines the two measurements. The two main advantages of ISM over fluorescence approaches are that absolute intracellular ion activity can be accurately recorded over a very large range of concentrations and calibration can be easily achieved before impalement of the cell then checked at the end of the experiment by simply dipping the electrode into saline containing different concentrations of the ion of interest.

Heiny et al. (2019) have brought the ISM technique to a high level of reliability and accuracy in enzymatically isolated mouse flexor digitorum brevis (fdb) muscle fibers using homemade ISM. The large size of the muscle cell allows four microelectrodes to be impaled, ensuring physiologically relevant measurements of intracellular Na^+, Cl^-, or H^+. Indeed, as mentioned above, the membrane potential is added to the voltage read by the ISM so that this voltage has to be subtracted to get actual measure of the ion activity. This is the function of the second intracellular microelectrode in charge of recording the membrane potential. Moreover, one two-barreled ISM or one single-barreled ISM plus one voltage electrode often causes membrane damage, generating a short that changes the membrane potential, and inducing currents that alter the ion activity being measured. In the technique described by Heiny et al. (2019), the measurement of ion activity with the ISM is not only separated from the measurement of membrane potential, but, by using two other intracellular microelectrodes, the membrane potential can be voltage clamped or adjusted to a desired value under current-clamp conditions. The function of these two additional microelectrodes is instrumental to a reliable measurement of intracellular activity by avoiding heavy impacts of membrane potential changes on intracellular ion activity and, reciprocally, by preventing the effect of large changes in ion concentrations on membrane potential and hence ion activity measurement. Recording the background current or cell membrane resistance by these two microelectrodes also gives the benefit of checking cell integrity all along the course of the experiment. The impalement of four microelectrodes can be viewed as additional injuries caused to the cell, but, by monitoring membrane potential and background current in the voltage clamp configuration, Heiny et al. (2019) provide evidence of very stable recording conditions without any signs of cell deterioration. It is remarkable that in close to 90% of preparations for which all four microelectrodes were successfully inserted, the background current, the membrane potential read using the ISM, and the calibration values of the ISM did not deviate significantly at the end of the experiment from their initial values. Additionally, Heiny et al. (2019) show that, although two of the four microelectrodes measure membrane potential at different locations, both electrodes measure very similar potentials. Therefore, for most purposes, the technique can be assuredly simplified to three electrodes: the single-barreled ISM and the two in charge of voltage clamp.

In their study, Heiny et al. (2019) first demonstrate the reliability of their method using a Na^+-selective ISM and show that half a second following a change in membrane potential (the response time of the ISM), the intracellular Na^+ activity remained, as expected, exactly the same as before the voltage pulse. Heiny et al. (2019) then display three conditions of intracellular ion activity changes, the first concerning Cl^- ions. Because Cl^- ions are mostly passively distributed across the muscle membrane, the Cl^- equilibrium potential is stabilized at a value close to the internal potential (Aickin et al., 1989). The high Cl^- conductance of the membrane implies that a change in membrane voltage leads to rapid transmembrane Cl^- movements until the Cl^- equilibrium potential meets the new value of membrane potential. This is exactly what Heiny et al. (2019) show by using a Cl^--selective ISM both in voltage- and current-clamp conditions. Interestingly, they validate their methods by showing that the Cl^- current produced by the voltage jump led to an amount of conducted Cl^- charges and a resulting change in concentration that exactly matches the change in concentration measured with the ISM. In a third series of experiments, they also demonstrate that a H^+-selective ISM monitors the expected intracellular acidification produced by exchange from a HEPES to a CO_2/bicarbonate buffered external solution in a cell held at −80 mV. Finally, using a Na^+-selective ISM, Heiny et al. (2019) highlight the full potential of their approach by measuring simultaneously, for the first time, the change in intracellular Na^+ together with the current generated by external K^+-induced changes in the activity of the Na/K ATPase in the presence of K^+, Cl^-, and Ca^{2+} channel inhibitors at resting potential.

In the study by Heiny et al. (2019), intracellular ion activity was explored in skeletal muscle fibers held at membrane potentials close to resting conditions. But this group has previously shown that space clamp is well maintained in short fdb fibers over the full range of physiologically relevant test potentials with the two-electrode voltage clamp technique (DiFranco et al., 2011). By taking the precaution of adding EGTA in the pipette used for voltage clamping in order to block contraction, it should thus be possible to measure ion activity in active muscle fibers developing action potentials or clamped at suprathreshold voltages. This valuable four-microelectrode method opens new perspectives for muscle physiology and pathophysiology. The technique is certainly effective for measuring absolute activity of Na^+, Cl^-, and H^+ and should be also applicable to K^+. The absolute changes of activity of these ions are poorly characterized at rest, in activity, and possibly during cell stretching and therefore deserve to be scrutinized. The use of this method should be also essential for exploring perturbations of ion activity that have been suggested to exist in a number of muscle channelopathies including periodic paralysis (Cannon, 2015; Allard and Fuster, 2018). Finally, one may hope that this study will contribute to the dissemination of muscle electrophysiology, which unfortunately has become scarce, and help muscle physiologists realize how critical it is to control voltage when investigating the movements of ions including the ubiquitous Ca^{2+} ion.

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