A better method to measure total calcium in biological samples yields immediate payoffs

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The calcium ion is the most universal and widely studied vector of chemical signals in living cells. Its presence and dynamic distribution in diverse organelles are associated with various functions, which include controlling protein expression and activity in response to functional demands. Calcium's role was first investigated in striated muscles, where it regulates contractile force. Muscles contain large amounts of calcium, most of which is selectively bound inside specific organelles. Its homeostasis and dynamics emerge from the precise interaction of various proteins, including channels of the plasmalemma and intracellular organelles, ion pumps, and both target and storage proteins. The large magnitude of the cyclic movements of calcium between the SR and cytosol requires an intra-SR calcium-binding molecule of large capacity and low affinity. These properties are found in calsequestrin, viewed not just as the main SR calcium-binding protein but also as a modulator of the pathway for calcium release. Cytosolic proteins, including troponin C and parvalbumin, as well as mitochondria, can also store substantial quantities of calcium.

Although great strides have been made in developing various reporters for measuring the concentration of the calcium ion and its dynamics in cytosol and organelles, the measurement of total steady calcium content in tissues has been, well, static, limited by the difficulties of the sole technique in wide use, namely atomic absorption spectroscopy (AAS; also known as flame photometry), which is done in supernatant solution after sample homogenization, denaturation, and removal of protein. Lamboley et al. (2015) now present a welcome alternative to AAS.

The new method relies on fluorescence of BAPTA

The first virtue of their new method is its simple rationale. It relies on the major shift in UV absorption spectrum of the calcium buffer BAPTA upon binding calcium. The authors delineate a technique that involves sample homogenization and removal of solids, and then derives Ca^{2+} concentration in the supernatant from the change in absorbance upon removal of the ion from BAPTA after the addition of EGTA. A salient design feature is the chosen concentration of BAPTA, calculated to bind essentially all calcium, while avoiding any excess that would reduce the dynamic range of the signal and hence the sensitivity of the method.

The paper is largely concerned with validating several assumptions that make the math simple and the process straightforward. Thus, the authors demonstrate that BAPTA ultimately binds all calcium present in the sample so that other ions do not affect or interfere with the amount of calcium-bound BAPTA, and that neither the intrinsic absorbance of the sample nor the binding of proteins to BAPTA interferes with the assay.

Given the general adherence of the results to these assumptions, the initial set of measures from various muscles of three animal species was narrowly distributed and allow for two- or three-figure precision in the averages—a major advance.

As for the actual calcium content, the numbers for mouse extensor digitorum longus (EDL; 2.71 ± 0.09; average ± SEM) and soleus (2.62 ± 0.11), in units of millimole per kilogram of muscle wet weight, are of most interest for current studies. The corresponding numbers for rat are about half of those for mice. These values are in substantial agreement with earlier results obtained with AAS in rats (Everts et al., 1993; Gissel and Clausen, 1999) but >50% greater than the earlier measurements in various mouse leg muscles.

Most calcium is in the SR

With simplifying assumptions, the authors can assert with some confidence that most of the Ca content of whole muscle is in the SR, with the rest distributed between cytosol-immersed molecules and extracellular space. (The estimate of calcium in the SR was 77% for EDL muscle, largely composed of fast-twitch fibers, and an even greater fraction in soleus, which lacks calcium-binding parvalbumin in the cytosol.) For the frog, these numbers translate to ∼2.5 mmoles/kg of myoplasmic water, in substantial agreement with the estimates of total releasable calcium in cut fibers under voltage clamp (Pape et al., 1995; Pizarro and Ríos, 2004).

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The large number of measurements included in the report suggests that the technique is simpler and more convenient than its predecessors. It is also more reproducible, as reflected in the small standard deviation of the results. This superior reproducibility may be related to the fact that the AAS readout is produced as the sample is being consumed; it is in this sense an intensive or differential measure, which reflects the concentration of the element in the sample. In contrast, the new method uses the change in absorption caused by the transfer of all the calcium content in the sample from one buffer to the other, essentially an extensive or integrating measure, which depends on content rather than concentration.

The new method brings immediate payoffs and a few surprises

Together with the apparent ease of use, greater accuracy, and consequent increase in precision of the novel technique come two sets of interesting observations.

The first is an intriguing inverse relationship between total calcium concentration [Ca_T] and muscle weight. This is not just a negative correlation between concentration and weight but a truly inverse relationship. In other words, the observed values $[Ca_T]_i$ (W_i), where W_i are wet weights of individual samples identified by the index i, are described well by

$$[Ca_T]_i = b\ W_i^{-1} + E_i, \quad (1)$$

where E_i is a random term representing measurement error. Observed values and their fit by Eq. 1 are represented in Fig. 1 A.

Lamboley et al. (2015) embraced Eq. 1 and its implications after ruling out an alternative interpretation, which can be deemed trivial; namely, that all samples have about the same calcium content. The alternative is best described with the equation resulting from multiplying both sides of Eq. 1 by W_i, an operation that yields amount of calcium in every sample:

$$[Ca_T]_i W_i = \text{Ca amount}_i = b + W_i E_i = b + E'_i. \quad (2)$$

The values resulting from this transformation are represented in Fig. 1 B.

In the interpretation preferred by the authors, embodied in Eq. 1, E is random, whereas E', equal to WE, is not. The alternative requires that E' be random (rather than E), and that the total amount of calcium in the samples be constant. In this view, the dependence of total calcium concentration on W is an artifact of the algorithm used to calculate concentration. This would be the case, for example, if individual muscles of similar weights and total calcium concentrations had been modified by variable water accumulation during the procedure. Inspection of the alternative plots of data and fits in Fig. 1 shows that one set of deviations is not visibly skewed, or worse than the other, suggesting that numeric analysis of errors will not help choose between these alternative possibilities.

The trivial alternative (Fig. 1 B) was ruled out by measurements of dry weight, which showed essential proportionality between dry and wet weight, thereby demonstrating an actual variation in muscle mass that was not simply the result of variable dilution by water. Therefore, the work has uncovered what appears to be a true inverse proportionality between muscle weight and total calcium concentration in skeletal muscle.

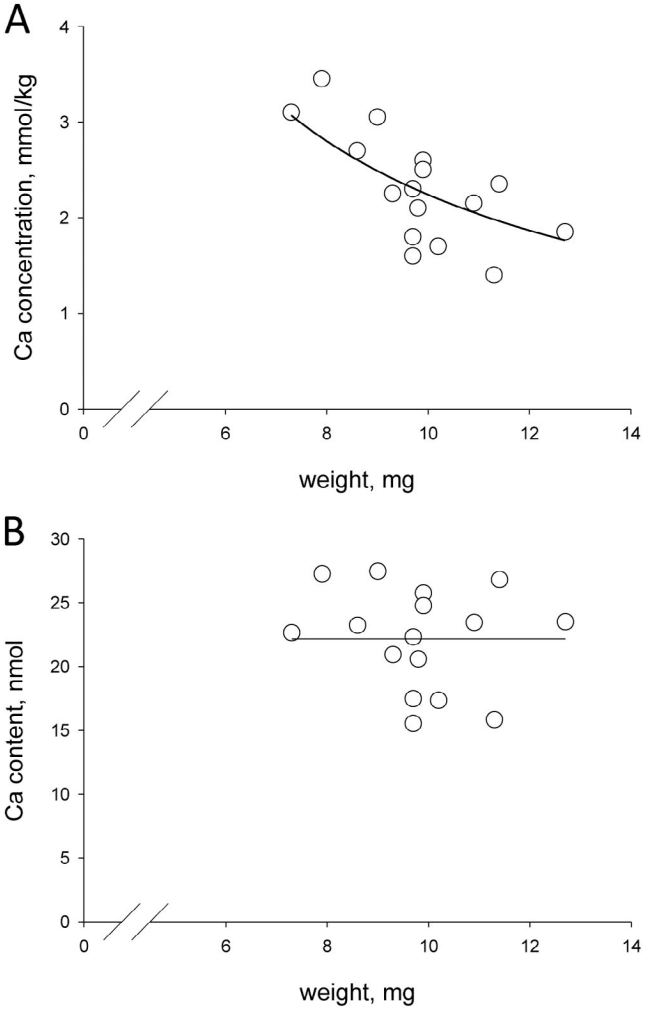

Figure 1. True inverse proportionality between [Ca_T] and muscle weight. (A) Values of total calcium concentration in EDL muscles of wild-type mice, as plotted in Fig. 7 A of Lamboley et al. (2015). The line is a best fit by the function represented in Eq. 1, with b = 22.4 nmol. (B) Total Ca content in individual muscles of A, calculated by multiplication of concentration and individual muscle weight. The line is traced at the average of all values (22.2 nmol). The fits in A and B represent alternative interpretations of the data; the one in A assumes an inverse relationship between total Ca concentration and weight, and the one in B assumes a total content that is independent of muscle weight. Lamboley et al. (2015) favor the first model, based on a separate comparison of muscle dry and wet weight.

Additionally, the authors report that these changes in muscle weight are not well correlated with total animal weight and propose a teleological explanation of the intriguing inverse relationship. According to their observations, smaller muscles face a greater demand for contractile tension (force per unit cross-sectional area). The extra calcium is presumed to be largely inside the SR, a condition conducive to a greater rate of calcium release and, at least under some circumstances, a greater tension.

Ablating the main Ca storage protein

An additional set of observations is relevant to our understanding of the calcium store for excitation–contraction coupling. They follow from measurements of total calcium content in muscles with no calsequestrin (with the calsequestrin 1 gene or the genes of both isoforms constitutively ablated). Calsequestrins 1 and 2 are, respectively, the main calcium-binding proteins in the SR of skeletal and cardiac muscle, and both have the low affinity required to release calcium rapidly and reversibly for contractile activation. There are, however, many unanswered questions regarding the magnitude and kinetics of calcium dissociation from calsequestrin, plus the suspicion that calsequestrin may play important calcium-dependent roles in regulating gating of the release channel (Royer and Ríos, 2009; Rebbeck et al., 2014).

In the present work, ablation of calsequestrin reduced total calcium concentration in the fast-twitch EDL muscle by 38%. This result, however, was reached after taking into account the substantial difference in weight between wild-type and calsequestrin-null muscles (a difference documented by Olojo et al., 2011). Indeed, the actual calcium concentration, in millimoles per kilogram of muscle, was not different between wild type and null. The 38% difference is the deficit found comparing concentration in the null with the higher value expected if the lighter null muscles satisfied the inverse concentration versus weight dependency established for wild-type muscle.

It is difficult to evaluate the accuracy and significance of this specific determination of reduction in total calcium content, because it involves extrapolating in two "dimensions," namely, an extrapolation to lower muscle weights not encountered in the wild type, and the assumption that the dependency encountered in the wild type should also apply to the genetically altered animals.

Regardless of this caveat, the remarkable message is that the differences (absolute or corrected for weight) are small, much less than expected for animals lacking the main SR calcium storage protein. This is particularly true of the results with soleus, where total calcium concentration is not reduced in null mice even after correction for the lower muscle weight (implying that the measured concentration, in millimoles per kilogram, is slightly greater in the nulls). The results, however, are consistent with a prior comparison in mouse flexor digitorum brevis that showed a decrease in releasable calcium by only 25% in the calsequestrin double null (Sztretye et al., 2011), as well as measurements that found essentially no change in releasable calcium in C2C12 skeletal myotubes (Wang et al., 2006) or in cardiomyocytes of a calsequestrin 2–null mouse (Knollmann et al., 2006).

Lamboley et al. (2015) explain the unexpectedly modest effect of calsequestrin ablation (a calcium deficit of 38% instead of the 75% predicted) as evidence of compensatory increase in the null muscle of other calcium-binding sites; these might include sites in at least four known SR proteins and on the SR membrane, which in the null has greater area (Paolini et al., 2007).

As the authors argue, compensation by increase in concentration of calcium-binding proteins in the calsequestrin-null mice is likely—and is incontrovertible in the case of the soleus. However, compensatory overexpression has not been quantified for specific proteins and might not constitute the only explanation for the present results. It is worth considering additional mechanisms, which may explain both the conservation of releasable calcium, observed in previous works, and the modest deficit in total calcium, now accurately measured by Lamboley et al. (2015).

The arguments for and against compensatory expression of Ca-binding proteins

The argument in favor of compensatory changes in calsequestrin-null mice requires two assumptions: one is that the ablation of calsequestrin does not change the resting value of free $[Ca^{2+}]_{SR}$, and the other is that essentially all bound calcium in the SR be bound to calsequestrin. Most of the cell calcium is in the SR and most of that is bound (a free fraction of 1/28 is the estimate used by Lamboley et al.; other estimates, as in Manno et al., 2013, are even lower). Under the above assumptions, it necessarily follows that any excess calcium in the null muscles must be bound to sites not present in the wild type.

Other explanations—which do not exclude the one preferred by the authors—require relaxing one or both assumptions. If free $[Ca^{2+}]_{SR}$ was greater in the calsequestrin-null muscle, releasable calcium would increase, perhaps more than proportionally, given the nonlinear properties of calsequestrin buffering (e.g., Royer et al., 2010; Fénelon et al., 2012). Consistent with this possibility, Sztretye et al. (2011) found an 18% greater average value for $[Ca^{2+}]_{SR}$ in cells of calsequestrin 1–null mice compared with those of wild-type mice (a difference that was not statistically significant). The observation of greater force and rate of force development in twitches of low frequency in calsequestrin-null mice points in the same direction (Olojo et al., 2011). In contrast, Canato et al. (2010) found no difference.

Better comparisons of resting $[Ca^{2+}]_{SR}$ between wild-type and calsequestrin-null muscles are required to elucidate this issue.

The assumption that all bound calcium inside the SR is bound to calsequestrin can also be questioned. Murphy et al. (2009) reported a concentration of calsequestrin of 1.4 g (or 29 µmol)/kg in rat EDL muscle. The same study concluded that calsequestrin contains 22% of its saturating calcium in resting cells. Assuming that rat calsequestrin 1 binds up to 80 Ca^{2+} per molecule (the highest of the values provided by Park et al., 2004, for rabbit and Sanchez et al., 2012, for human calsequestrin), the resting calcium content of calsequestrin in the rat would be 0.51 mmol/kg. The present measurements put the calcium content of rat EDL at rest at 1.46 mmol/kg, 77% of which (1.12 mmol/kg) are in the SR. Clearly, the measured amounts of calsequestrin content are too low to match the present measure of resting calcium content.

The estimated calcium content of calsequestrin is likewise insufficient to account for current estimates of releasable calcium. For the frog (Pape et al., 1995), these are ∼2.6 mmole/liter of cytosolic water, or 1.46 mmole/kg of muscle. As Lamboley et al. point out, the various estimates of releasable calcium in the literature are largely consistent with the total calcium content now reported. These numbers, however, seem substantially greater than the above estimates of calcium bound to calsequestrin, leaving substantial room for additional sources of calcium in wild-type muscle. (Other evidence of these has existed for a long time. Shortly after calsequestrin was revealed as the main Ca-binding protein in the SR, raw fractionation data of proteins of the SR were published, consistent with the existence of substantial acidic Ca-binding proteins other than calsequestrin [MacLennan et al., 1972]. The presence of luminal ER proteins capable of binding large amounts of calcium was later stressed by MacLennan and Reithmeier, 1998).

A compensatory increase in calcium-binding proteins should ideally be detected by accurate quantification of the overexpressed proteins. But this question can also be addressed with the new method. The putative compensation should be limited when the abundance of the protein is reduced via acute gene silencing instead of gene ablation. Silencer shRNA drastically reduced the endowment of calsequestrin 1 in mouse muscles within 1 wk of application (Royer, 2009; Royer et al., 2009). The reduction in total calcium concentration in muscles treated thus, which can now be measured precisely using the method of Lamboley et al. (2015), should reflect the amount originally bound to calsequestrin.

Other applications

Many other questions could be effectively addressed with the new technique. A general issue with multiple ramifications is the quantitative contribution of plasmalemmal entry of calcium to sustain or modify calcium load and contractility under various conditions (say in health, various diseases, or aging). For example, in most animal models of malignant hyperthermia (MacLennan and Zvaritch, 2011) and in some patients, myoplasmic free $[Ca^{2+}]$ is elevated, a change ultimately caused by alterations in plasmalemmal calcium transport (e.g., Eltit et al., 2012). The new technique will support new ways to quantify how much alteration in plasmalemmal calcium entry is needed and whether or not it can be inhibited as a possible approach to preventing malignant hyperthermia events.

To close, we stress another cause to celebrate the contribution by Lamboley et al. (2015); as claimed by the authors, this method, with suitable adaptations, should be applicable to many other tissues and organs. This, of course, is its greatest promise.

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