

Zinc flexes its muscle: Correcting a novel analysis of calcium for zinc interference uncovers a method to measure zinc

Cheng Qian and Robert A. Colvin

Department of Biological Sciences, Neuroscience Program, Ohio University, Athens, OH 45701

The divalent cation chelator 1,2-bis(o-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid (BAPTA), often used to buffer physiological changes in cytosolic Ca^{2+} , also binds Zn^{2+} with high affinity. In a recently published method (Lamboley et al. 2015. *J. Gen. Physiol.* <http://dx.doi.org/10.1085/jgp.201411250>), the absorbance shift of BAPTA at 292 nm was successfully used to determine the total calcium concentrations of various skeletal muscle tissues. In the present study, we show that endogenous Zn^{2+} in rat skeletal muscle tissue can be unknowingly measured as “ Ca^{2+} ,” unless appropriate measures are taken to eliminate Zn^{2+} interference. We analyzed two rat skeletal muscle tissues, soleus and plantaris, for total calcium and zinc using either inductively coupled plasma mass spectrometry (ICP-MS) or the BAPTA method described above. ICP-MS analysis showed that total zinc contents in soleus and plantaris were large enough to affect the determination of total calcium by the BAPTA method (calcium = 1.72 ± 0.31 and 1.96 ± 0.14 , and zinc = 0.528 ± 0.04 and 0.192 ± 0.01 ; mean \pm standard error of the mean [SEM]; $n = 5$; mmole/kg, respectively). We next analyzed total calcium using BAPTA but included the Zn^{2+} -specific chelator *N,N,N',N'*-tetrakis(2-pyridylmethyl)ethylenediamine (TPEN) that buffers Zn^{2+} without affecting Ca^{2+} /BAPTA binding. We found that estimated concentrations of total calcium ($[\text{Ca}_T]_{\text{WM}}$) in soleus and plantaris were reduced after TPEN addition ($[\text{Ca}_T]_{\text{WM}} = 3.71 \pm 0.62$ and 3.57 ± 0.64 without TPEN and 3.39 ± 0.64 and 3.42 ± 0.62 with TPEN; mean \pm SEM; $n = 3$; mmole/kg, respectively). Thus, we show that a straightforward correction can be applied to the BAPTA method to improve the accuracy of the determination of total calcium that should be applicable to most any tissue studied. In addition, we show that using TPEN in combination with the BAPTA method allows one to make reasonable estimates of total zinc concentration that are in agreement with the direct determination of zinc concentration by ICP-MS.

INTRODUCTION

In a series of well-documented experiments, Lamboley et al. (2015) recently describe an elegant method for measuring total calcium concentrations in skeletal muscle tissues based on a well-characterized calcium-dependent absorbance shift (at 292 nm) of the frequently used divalent ion chelator 1,2-bis(o-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid (BAPTA). The authors were able to determine the concentrations of total calcium in several different skeletal muscle tissues. Although BAPTA can bind divalent cations other than Ca^{2+} , the method works because other physiologically relevant divalent cations have either a much lower binding affinity for BAPTA than Ca^{2+} , or total levels are too small to interfere with Ca^{2+} /BAPTA binding. This assumption (no. 2 in Lamboley et al., 2015) was verified for Mg^{2+} (binding affinity for Mg^{2+} /BAPTA is five orders of magnitude weaker than that of calcium; Tsien, 1980) and protons, and therefore assumed to be the case for other potentially interfering ions. In contrast, Zn^{2+} is well known to compete with calcium for numerous calcium-binding sites (e.g., tubulin, mitochondrial Ca^{2+} transporter,

DNA, and various calcium indicators; Jeng et al., 1978; Jemiolo and Grisham, 1982; Waalkes and Poirier, 1984; Martin et al., 2006). Unlike Mg^{2+} , Zn^{2+} does bind to BAPTA with high affinity (Csermely et al., 1989); the K_d for Zn^{2+} /BAPTA binding has been reported in several sources to be 7.9 nM (Benters et al., 1997; compare with Ca^{2+} /BAPTA binding, $K_d = 110$ nM; Tsien, 1980). Although resting cytosolic free Zn^{2+} is >100-fold less than resting cytosolic free Ca^{2+} (Colvin et al., 2008, 2010), total zinc tissue levels are 1/10 to 1/3 of total calcium levels (Colvin et al., 2015). Because total levels of zinc are not negligible compared with total calcium in tissues, and because BAPTA binds Zn^{2+} with much greater affinity, one would expect measurable interference from endogenous Zn^{2+} when total calcium is determined using the BAPTA method (Lamboley et al., 2015).

We hypothesized that Zn^{2+} interference of the BAPTA method to measure total calcium should be observable if endogenous Zn^{2+} could be eliminated without affecting Ca^{2+} /BAPTA binding. Fortunately, transition metal chelators are available with high affinity for Zn^{2+} , but

Correspondence to Robert A. Colvin: colvin@ohio.edu

Abbreviations used in this paper: BAPTA, 1,2-bis(o-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid; ICP-MS, inductively coupled plasma mass spectrometry; TPEN, *N,N,N',N'*-tetrakis(2-pyridylmethyl)ethylenediamine.

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they bind much less selectively to Ca^{2+} . *N,N,N',N'*-tetrakis(2-pyridylmethyl)ethylenediamine (TPEN) is one such molecule with high affinity for zinc ($K_d = 10^{-15.58}$ M) and iron ($K_d = 10^{-14.61}$ M), but it shows much lower affinity for calcium ($K_d = 10^{-2.44}$ M) and magnesium ($K_d = 10^{-1.7}$ M) (Arslan et al., 1985). In the present study, we first tested the hypothesis that total zinc levels in rat skeletal muscle tissues are in a range that would predict interference with the BAPTA-based total calcium determination. Next, we demonstrated that the Zn^{2+} chelator TPEN effectively binds Zn^{2+} without affecting the Ca^{2+} /BAPTA interaction and can be used with the BAPTA method to eliminate Zn^{2+} interference in the determination of total calcium. Finally, we show that combining TPEN with the BAPTA method allows one to make a reasonable estimate of the total zinc concentration of the tissue.

MATERIALS AND METHODS

Chemical reagents and solution preparation

The measurement solution—120 mM NaCl (VWR International) and 2 mM HEPES (Sigma-Aldrich) with 0.5% (wt/vol) SDS (Thermo Fisher Scientific)—was adjusted to pH 8.0, as recommended by Lamboley et al. (2015). Notably, the term “measurement solution” in this paper means the solution used as the measuring medium, but it does not contain BAPTA. A 4-mM BAPTA (Sigma-Aldrich) stock solution was prepared in 0.3 N NaHCO_3 (Thermo Fisher Scientific) to reach an optimal solubility (50 mg/ml) as suggested by Sigma-Aldrich. The TPEN (Santa Cruz Biotechnology, Inc.) stock solution was made as 20 mM in DMSO. ZnCl_2 (Thermo Fisher Scientific) was prepared as either 10 or 25 mM in aqueous solution. A calcium standard solution (1,000 mg/L \pm 4 mg/ml; Fluka Analytical) was purchased from Sigma-Aldrich.

Animals and tissue preservation

Male Fischer 344 rats from the National Institutes of Health Aging Colony (Harlan Laboratories, Inc.) were anaesthetized with xylazine and ketamine, and soleus and plantaris muscles were removed. Tissues were mounted on flat sticks for histology studies using a mixture of tragacanth gum and OCT freezing compound, frozen in methyl butane chilled with liquid nitrogen, and then stored in sealed vials in -80°C (Brooks et al., 2009). All procedures involving animals were approved by the Ohio University Institutional Animal Care and Use Committee.

Inductively coupled plasma mass spectrometry (ICP-MS) detection of total calcium and zinc in skeletal muscle tissues

Approximately 5-mg samples were taken from rat soleus and plantaris muscles (see Animals and tissue preservation above) after thawing and digested in concentrated nitric acid as described previously (Colvin et al., 2015). ICP-MS analysis was performed at The Ohio State University Trace Element Laboratory using a Finnigan ELEMENT 2 instrument (Thermo Fisher Scientific). Values were corrected for instrument drift using internal standards (Sc^{45} , Y^{89} , and In^{115}) added at 10 ppb.

Absorbance spectra of TPEN, BAPTA, BAPTA/Zn and BAPTA/Zn/TPEN

The spectrophotometer (Cary 50 UV-Visible; Agilent Technologies) was set to scan absorbance from 250 to 350 nm. The spectrophotometer was zeroed with an empty quartz cuvette (1 cm optical

distance). TPEN and BAPTA were added to the measurement solution to obtain the desired final concentrations. Extinction coefficients for Ca^{2+} /BAPTA and Zn^{2+} /BAPTA with or without the addition of TPEN were obtained by the step-wise addition of Ca^{2+} standard or ZnCl_2 to obtain 0.01-, 0.025-, 0.05-, 0.075-, 0.1-, 0.15-, and 0.2-mM final concentrations.

BAPTA measurement of total calcium levels in rat skeletal muscles in the presence or absence of TPEN

The measurement solution (NaCl/HEPES/SDS, without BAPTA) was used to homogenize skeletal muscle tissues instead of the KCl/HEPES/SDS solution used by Lamboley et al. (2015) because SDS has better solubility properties in NaCl. Samples of skeletal muscle tissues (\sim 35 mg) were completely thawed on ice and carefully removed from the mounting media. The rat skeletal muscle tissue was first minced with a scalpel with a fresh blade, and then transferred into a 5-ml straight shaft tissue grinder (Pyrex) which was pre-rinsed with 1 ml of measurement solution (120 mM NaCl and 2 mM HEPES, 0.5% SDS). 5 ml of measurement solution was added into the grinder. After settling down, the rat skeletal muscle tissue was homogenized (\sim 10 up/down strokes) until the sample formed a homogeneous appearance without visible particulates. The tissue homogenate and blank measurement solution were first aliquoted into four 1.5-ml microcentrifuge tubes, respectively, and then centrifuged at 13,000 rpm for 45 min at 6°C . 900 μl of supernatant was recovered for later measurement. Muscle supernatant and measurement solution were first scanned to obtain a background without BAPTA addition. Next, BAPTA was added from a 4-mM stock solution to obtain a final concentration of 0.15 mM in the muscle supernatant or measurement solution. TPEN was added from a 20-mM stock solution (in DMSO) to obtain a final concentration of 0.2 mM in the muscle homogenate or measurement solution after BAPTA addition. Data acquisition and calculations to determine concentrations of total calcium or zinc are described in greater detail in the Results and Discussion.

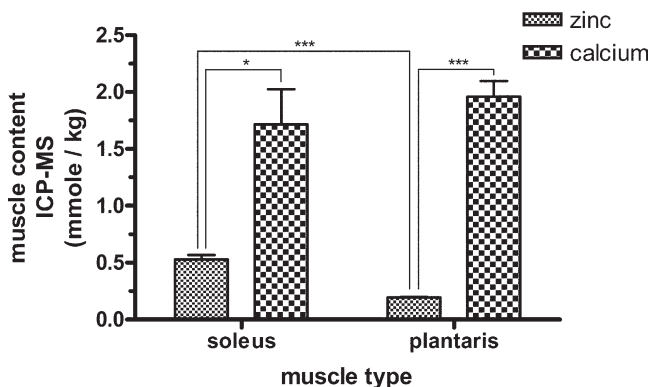


Figure 1. Total concentration of zinc or calcium was determined in acid digests of rat soleus or plantaris muscle tissue by ICP-MS. Results are expressed in mmole/kg (muscle wet weight). *, significantly different when Zn^{2+} is compared with Ca^{2+} in the soleus muscle; $P < 0.05$; Student's unpaired *t* test. ***, significantly different when Zn^{2+} is compared with Ca^{2+} in the plantaris muscle, and when Zn^{2+} in the soleus is compared with Zn^{2+} in the plantaris; $P < 0.0001$; Student's unpaired *t* test. No significant difference was found when comparing Ca^{2+} in the soleus with Ca^{2+} in the plantaris muscle. $n = 5$ for each muscle type. Error bars represent mean \pm SEM.

Statistical analysis and computer programs

Data analysis and graphing were aided by the use of GraphPad Prism (version 4.03 for Windows; GraphPad Software). Two-way ANOVA, Student's unpaired *t* test, and linear regression analyses were performed using the GraphPad Prism software, and results were judged significantly different when $P \leq 0.05$. Two-way ANOVA was used to compare the means obtained for calcium concentration before and after TPEN addition, in either soleus or plantaris muscles (Table 1), and to compare the means obtained for zinc concentration estimated by the simple subtraction and corrected simple subtraction, in either soleus or plantaris muscles (Table 2). A Student's unpaired *t* test was used to compare the means obtained for zinc and calcium concentration, in soleus and plantaris, measured with ICP-MS (Fig. 1). Calculation of the cytosolic free zinc and calcium concentrations in the presence of multiple chelators was aided by the use of WINMAXC32 (version 2.51 for Windows).

RESULTS

ICP-MS analysis indicates that total zinc levels in rat skeletal muscles are large enough to potentially interfere with Ca^{2+} /BAPTA binding

ICP-MS analysis of acid-digested rat skeletal muscle tissues was used to directly measure total tissue zinc and calcium concentration (Fig. 1). These samples were taken from the same rat skeletal muscle tissues used for BAPTA total calcium measurements described below. We found that soleus and plantaris muscle total calcium concentrations determined by ICP-MS (1.72 ± 0.31 and 1.96 ± 0.14 ; mean \pm SEM; $n = 5$, mmole/kg, respectively) were similar to values obtained by Lamboley et al. (2015), confirming the validity of this method to measure total tissue calcium. Most importantly, ICP-MS analysis (Fig. 1) confirmed that total zinc levels in rat skeletal muscle (soleus, 0.53 ± 0.04 ; plantaris, 0.19 ± 0.01 ; mean \pm SEM; $n = 5$; mmole/kg) were large enough to potentially interfere with the determination of total calcium in rat muscle homogenates when using the BAPTA method.

Spectrophotometric analysis of TPEN and its ability to completely reverse the Zn^{2+} -induced BAPTA absorbance shift at 292 nm

TPEN, to effectively eliminate Zn^{2+} and be used to correct Zn^{2+} interference with the Ca^{2+} /BAPTA interaction, must have three critical properties. First, TPEN must have low intrinsic absorbance at 292 nm; second, TPEN must be able to completely reverse the BAPTA absorbance shift at 292 nm caused by Zn^{2+} binding; and third, TPEN must not interfere with the Ca^{2+} /BAPTA interaction at physiologically relevant concentrations. Fig. 2 A shows that the intrinsic absorbance at 292 nm of 0.2 mM TPEN is low (0.15 above the background absorbance determined with the measurement solution) and acceptable for use in the BAPTA measurement of total calcium. Fig. 2 A shows as well that when TPEN and BAPTA are added together, the small TPEN intrinsic absorbance produces a small shoulder in the BAPTA

absorbance at 292 nm, similar in size to that observed with TPEN alone. Next, we show that when 0.15 mM ZnCl_2 was added to 0.15 mM BAPTA in the measurement solution, BAPTA absorbance decreased as expected (Fig. 2 B), and the decrease in BAPTA absorbance induced by Zn^{2+} addition was reversed by the addition of 0.2 mM TPEN (Fig. 2 B). This experiment clearly shows that Zn^{2+} interference can be effectively eliminated by TPEN even in the presence of BAPTA.

Finally, Fig. 2 C shows that the addition of TPEN does not interfere with Ca^{2+} /BAPTA binding, as seen by almost identical slopes of the Ca^{2+} /BAPTA and Ca^{2+} /BAPTA/TPEN data. Here, we show analysis of the Ca^{2+} /BAPTA and Zn^{2+} /BAPTA extinction coefficients with or without the addition of 0.2 mM TPEN. The linear portion of the Ca^{2+} -induced BAPTA absorbance shift at 292 nm was used to calculate the extinction coefficient for Ca^{2+} /BAPTA binding ($\Delta\epsilon = -4.63 \text{ mM}^{-1} \cdot \text{cm}^{-1}$; Fig. 2 C). The linear portion of Zn^{2+} -induced BAPTA absorbance shift at 292 nm nearly superimposed with the Ca^{2+} /BAPTA data, yielding a similar extinction coefficient ($\Delta\epsilon = -3.98 \text{ mM}^{-1} \cdot \text{cm}^{-1}$; Fig. 2 C). Next, these experiments were repeated in the presence of 0.2 mM TPEN (Fig. 2 C). An increase in absorbance was observed before the addition of either Ca^{2+} or Zn^{2+} corresponding to the intrinsic absorbance of 0.2 mM TPEN at 292 nm (see Fig. 2 A). The calculated extinction coefficient for Ca^{2+} /BAPTA binding in the presence of TPEN was slightly less than that calculated without TPEN present ($\Delta\epsilon = -4.31 \text{ mM}^{-1} \cdot \text{cm}^{-1}$; Fig. 2 C). In contrast, when Zn^{2+} was added in the presence of 0.2 mM TPEN, the ability of TPEN to effectively bind and eliminate Zn^{2+} binding to BAPTA was clearly demonstrated ($\Delta\epsilon = -0.25 \text{ mM}^{-1} \cdot \text{cm}^{-1}$; Fig. 2 C). In addition, the intrinsic absorbance of TPEN was not sensitive to the addition of either Ca^{2+} or Zn^{2+} (not depicted).

Demonstration of how TPEN addition can be used to correct for Zn^{2+} interference of Ca^{2+} /BAPTA binding

Fig. 3 (A and B) shows representative absorbance scans for rat skeletal muscle tissue homogenates, illustrating the procedure developed to use TPEN addition to correct Zn^{2+} interference of Ca^{2+} /BAPTA binding. Fig. 3 A shows the analysis using the blank measurement solution only. First, BAPTA is added to obtain the absorbance with only BAPTA present (S_M). To the BAPTA solution, 1 mM EGTA is added to eliminate all Ca^{2+} -BAPTA binding (S_0). It can be seen that little if any shift in absorbance was observed, indicating that contaminating Ca^{2+} was insignificant in the measurement solution. In a second sample, 1 mM Ca^{2+} was added (S_∞), which provides the full range of BAPTA absorbance shift ($S_\infty - S_0$) under these conditions. Based on the calculation published by Lamboley et al. (2015), these parameters are used to calculate the $f_{\text{background}}$, which is the ratio of BAPTA absorbance shift caused by Ca^{2+} in the blank

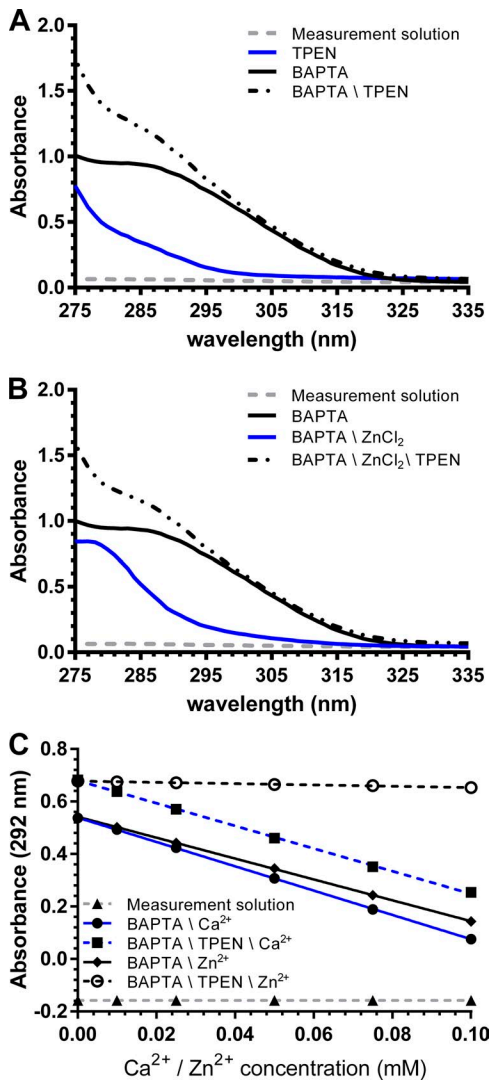


Figure 2. TPEN and BAPTA spectrophotometric analysis. BAPTA absorbance shift after Zn^{2+} or Ca^{2+} binding in the presence or absence of TPEN was determined by spectrophotometric analysis (250–350-nm scan). (A) The spectrum of 0.2 mM TPEN in the measurement solution shown as the blue solid line. The spectrum of 0.15 mM BAPTA in the measurement solution shown as the black solid line. The combination of 0.15 mM BAPTA and 0.2 mM TPEN spectrum shown as the black dash/dot line. (B) The addition of 0.15 mM ZnCl_2 into 0.15 mM BAPTA in the measurement solution caused a reduction in BAPTA absorbance (blue solid line) compared with the unbound BAPTA spectrum around 292 nm (black solid line). The addition of 0.2 mM TPEN into the Zn^{2+} -bound BAPTA yielded a spectrum shown as the black dash/dot line. (C) Determination of the extinction coefficient ($\Delta\epsilon$) for Ca^{2+} or Zn^{2+} binding to BAPTA: Ca^{2+} standard or ZnCl_2 was added step-wise to the 0.15-mM BAPTA solution to obtain 0.01-, 0.025-, 0.05-, 0.075-, 0.1-, 0.15-, and 0.2-mM final concentrations. The Ca^{2+} and Zn^{2+} additions to BAPTA were repeated twice. Determination of $\Delta\epsilon$ for Ca^{2+} or Zn^{2+} binding to BAPTA in the presence of TPEN: TPEN stock solution was added into 0.15 mM BAPTA in the measurement solution to reach a final concentration of 0.2 mM. Ca^{2+} standard or ZnCl_2 was added step-wise to the 0.15-mM BAPTA/0.2-mM TPEN solution to obtain 0.01-, 0.025-, 0.05-, 0.075-, 0.1-, 0.15-, and 0.2-mM final concentrations. The Ca^{2+} and Zn^{2+} additions to 0.15 mM BAPTA/0.2 mM TPEN were repeated

measurement solution over the full range of BAPTA absorbance shift ($f_{\text{background}} = (S_M - S_0)/(S_Z - S_0)$; i.e., we obtained $f_{\text{background}} = 0.058$ in this representative measurement solution). Next, these same procedures were repeated in the presence of 0.2 mM TPEN to obtain $S_{M,T}$, $S_{0,T}$, and $S_{Z,T}$. The absorbance spectrum of BAPTA was unchanged except for a small absorbance shift for each parameter (S_M , S_0 , and S_Z) resulting from the intrinsic absorbance of 0.2 mM TPEN (Fig. 3 A; see also Fig. 2). These data are used to correct for the contribution of the intrinsic absorbance of TPEN in the calculation of total calcium. In the presence of TPEN, this representative blank solution gave $f_{\text{background,T}} = 0.052$. Thus, TPEN addition did not interfere with the calculation of background f value.

Muscle homogenates were split into two samples: one that would be used to determine total calcium content without TPEN addition, and a second used to correct the first determination for Zn^{2+} interference by the addition of TPEN (see Fig. 3 B). The addition of 1 mM EGTA (A_0) to the BAPTA-muscle homogenate (A_M) resulted in a measurable absorbance shift, presumably caused by the chelation of both Ca^{2+} and Zn^{2+} in the muscle homogenate. 1 mM Ca^{2+} was added to obtain the full range of BAPTA absorbance shift ($A_Z - A_0$) needed for the calculation of total calcium. Similar to the calculation of $f_{\text{background}}$ for the blank measurement solution, the f value of muscle homogenate can be calculated based on these parameters ($f = (A_M - A_0)/(A_Z - A_0)$; i.e., we obtained $f = 0.280$ in this representative soleus muscle). The second sample (same muscle homogenate) was treated similarly except that 0.2 mM TPEN was included throughout (Fig. 3 B) to obtain $A_{M,T}$. The small shift in absorbance caused by the intrinsic absorbance of TPEN can be observed, and EGTA addition ($A_{0,T}$) still results in an absorbance shift. Presumably, this absorbance shift induced by EGTA represents Ca^{2+} only, as the available Zn^{2+} in the muscle homogenate has been eliminated by the addition of TPEN. As above, in a separate sample, Ca^{2+} is added to obtain $A_{Z,T}$, the full range of BAPTA absorbance shift needed for the calculation. When comparing the data with or without TPEN addition in this example, TPEN addition resulted in $f_T = 0.223$ (see above; $f = 0.280$ before TPEN addition, which is the fraction of BAPTA bound with Ca^{2+} and Zn^{2+} ; f_T is the fraction of BAPTA bound with Ca^{2+} only). Presumably, the change in f value after TPEN addition is proportional to the total zinc concentration of the muscle homogenate.

twice. Values of BAPTA absorbance at 292 nm were plotted using GraphPad Prism software as a function of increasing Ca^{2+} or Zn^{2+} concentration. The linear portion of the additions (0–0.10 mM) was used to estimate the extinction coefficients by performing linear regression analysis.

To calculate the actual concentration of total calcium in the muscle tissue without including calcium in the measurement medium, $f_{\text{background}}$ or $f_{\text{background,T}}$ is first subtracted from f or f_T to obtain f_M or $f_{M,T}$, respectively. The stoichiometry of Ca^{2+} binding to BAPTA is 1:1; therefore, the concentration of Ca^{2+} in the muscle homogenate $[\text{CaB}]_M$ can be calculated as $f_M \times [\text{B}_T]$. Specifically, $[\text{B}_T]$ is determined from the full range of BAPTA absorbance shift ($A_\infty - A_0$) and the calculated extinction coefficient of BAPTA for Ca^{2+} ($\Delta\epsilon = -4.63 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ obtained from Fig. 2). Similarly, $[\text{B}_T]_{\text{TPEN}}$ of the TPEN-treated muscle homogenate solution is determined from full range shift ($A_{\infty,T} - A_{0,T}$) and the calculated extinction coefficient of BAPTA/TPEN for Ca^{2+} ($\Delta\epsilon = -4.31 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ obtained from Fig. 2). As stated above, both Ca^{2+} and Zn^{2+} in the muscle bind to BAPTA. Thus, Eq. 16a in Lamboley et al. (2015) should be modified as the following in the absence of TPEN adjustment:

$$[\text{CaB}]_M + [\text{ZnB}]_M = f_M \cdot [\text{B}_T]. \quad (1)$$

After TPEN addition, the BAPTA absorbance shift is caused only by Ca^{2+} binding and can be expressed as:

$$[\text{CaB}]_M = f_{M,T} \cdot [\text{B}_T]_{\text{TPEN}}. \quad (2)$$

The final result $[\text{Ca}_T]_{\text{WM}}$ is calculated after normalizing the measured concentrations of total calcium to the wet weight of muscle tissues and the volume of the muscle homogenate. Table 1 shows the results of such an analysis conducted on three individual samples of rat soleus and plantaris muscle taken from six different animals. The calculations shown in Table 1 were obtained following the procedures outlined above for muscle homogenates analyzed with or without the addition of TPEN (Lamboley et al., 2015). Listed in Table 1 are the average wet weights of the soleus and plantaris muscle tissues used for the analysis and the average concentrations of total calcium determined with and without TPEN addition. In each muscle tissue sample assayed, we found that the calculated concentrations of total calcium $[\text{Ca}_T]_{\text{WM}}$ were reduced by TPEN addition. The most likely explanation for this observation was that Zn^{2+} present in the muscle homogenates was interfering with the Ca^{2+} /BAPTA binding determination and that after TPEN addition, Zn^{2+} interference was effectively eliminated.

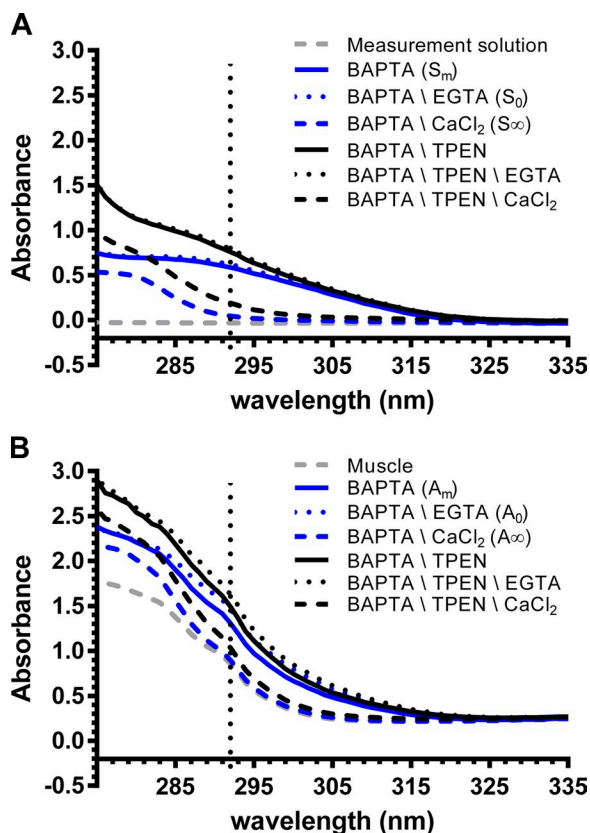


Figure 3. Representative absorbance scans obtained for total calcium and zinc determinations using the BAPTA method. See Results for additional explanation. This particular example is from rat soleus muscle tissue homogenate and yielded the following values for total calcium and zinc concentrations (2.738 mmole/kg Ca^{2+} without TPEN addition; 2.348 mmole/kg Ca^{2+} with TPEN addition). (A) Blank measurement solution. (B) Rat soleus muscle tissue homogenate. 0.15 mM BAPTA, 1 mM EGTA, 1 mM CaCl_2 , and 0.2 mM TPEN were used in the corresponding trials. A vertical dotted line is used to help visualize the absorbance values at 292 nm.

TABLE 1

BAPTA determination of concentrations of total calcium in rat skeletal muscle tissues with or without the addition of TPEN

Muscle Type	Wet weight mg	$[\text{Ca}_T]_{\text{WM}}$ mmole/kg	
		Without TPEN ^a	With TPEN ^a
Soleus	31.7 ± 7.80	3.71 ± 0.623	3.39 ± 0.635
Plantaris	37.3 ± 10.6	3.57 ± 0.643	3.42 ± 0.624

The wet weight of different rat skeletal muscle tissues expressed in mg and the calculated $[\text{Ca}_T]_{\text{WM}}$ expressed in mmole/kg are summarized here. Mean ± SEM.

^aTwo-way ANOVA was conducted on four groups of $[\text{Ca}_T]_{\text{WM}}$ values measured with or without TPEN addition in soleus or plantaris to determine which variable—TPEN addition (variable 1) and/or muscle types (variable 2)—was significant. TPEN addition (variable 1) was found to be significant ($P < 0.01$); $n = 3$ for each muscle type. No significant difference was found for muscle type.

DISCUSSION

Measurement of tissue concentrations of total calcium and zinc

In this study, we developed a method using TPEN addition to effectively eliminate Zn^{2+} interference and increase the accuracy of the BAPTA method used to determine total calcium in rat skeletal muscle tissues (Lamboley et al., 2015). We arrived at these conclusions

by comparing results of the BAPTA method (with or without TPEN addition) with those obtained by the direct method of analyzing the same muscle tissues for zinc and calcium concentrations using ICP-MS analysis. We recommend highly that TPEN be applied routinely when the BAPTA method is used to calculate the total calcium concentration of any tissue sample. The Zn^{2+} interference observed for the rat skeletal muscle tissues in the present studies is probably fairly representative of the total zinc content of most mammalian tissues (Colvin et al., 2015) and may vary concurrently with physiological manipulations intended to manipulate or alter the calcium content of the tissue being studied. In such situations, the results of the BAPTA assay may erroneously report changes in calcium concentration that actually reflect changes in zinc concentration or fail to detect changes in calcium concentration because zinc concentration is altered in an opposite direction. In these situations, the error introduced by zinc may be greater than the small effect we observed in resting skeletal muscle tissues. In our studies, we used a large excess of TPEN to assure complete chelation of Zn^{2+} in the muscle homogenate. Based on the amount of zinc in tissues relative to calcium and the affinity of TPEN for zinc, we suspect that lower concentrations of TPEN could be used to eliminate Zn^{2+} interference when using the BAPTA method.

A notable discrepancy was observed between our results for total calcium using the BAPTA method and our results from ICP-MS or those published by Lamboley et al. ($[Ca_T]_{WM} = 3.71$ and 3.57 mmole/kg for soleus and plantaris, our BAPTA method, respectively; 1.72 and 1.96 mmole/kg for soleus and plantaris, our ICP-MS analysis, respectively, and 1.26 and 1.46 mmole/kg for rat soleus and EDL; Lamboley et al., 2015, respectively).

TABLE 2

Determination of concentrations of total zinc in rat skeletal muscle tissues using TPEN in combination with the BAPTA method

Muscle type	Simple subtraction $[Zn_T]_{WM}$	Corrected simple subtraction $[Zn_T]_{WM,corrected}$
	<i>mmole/kg^a</i>	<i>mmole/kg^a</i>
Soleus ^b	0.319 ± 0.046	0.370 ± 0.054
Plantaris ^b	0.152 ± 0.031	0.176 ± 0.036

The normalized concentrations of total zinc ($[Zn_T]_{WM}$ or $[Zn_T]_{WM,corrected}$; mmole/kg) estimated with or without correction using either soleus or plantaris muscle tissues are summarized here. Mean \pm SEM. Two-way ANOVA was conducted on four groups of concentration of total zinc values using the simple subtraction or corrected simple subtraction, in either soleus or plantaris to determine which variable—the correction made on the simple subtraction (variable 1) and/or muscle types (variable 2)—was significant.

^aThe correction calculation (variable 1) was found to be significant ($P < 0.01$).

^bThe muscle type (variable 2) was also found to be significant ($P < 0.05$); $n = 3$ for each muscle type.

We can think of three possible explanations for this discrepancy, two of which relate to the fact that the tissue samples used for this analysis were originally prepared by Brooks et al. (2009) for histological analysis. The tissue samples were mounted and frozen for ease of later cryostat sectioning. Each tissue used here had been sectioned but was kept frozen until our study. The smaller samples (5 mg) taken for ICP-MS analysis were visually selected to be pure muscle tissue and not contaminated by the mounting medium. On the other hand, for the BAPTA method analysis, we used all the remaining tissue on the wooden stick. This included the muscle tissue proper but also may have included muscle tendons and connective tissue not present in the sample for ICP-MS analysis. It is plausible that muscle tendons and connective tissue may contain much higher levels of calcium (wet weight basis) than the muscle tissue proper contributing to the higher overall values for $[Ca_T]_{WM}$ determined in the present study. Although beyond the scope of this study, it will be interesting to determine if muscle tendons and connective tissues have significantly different levels of zinc and calcium than the muscle tissue proper. In addition, we cannot rule out the possibility of Ca^{2+} contamination of the tissue samples coming from the mounting medium. The third explanation is that the Pyrex tissue grinder used to homogenize the muscle tissue could be a source of calcium contamination (see Lamboley et al., 2015, section 4 of the supplemental text regarding potential calcium contamination from labware). This may explain why our ICP-MS calcium estimates matched more closely with Lamboley et al., as the samples for ICP-MS were all acid-digested in plastic microcentrifuge tubes. Our experiments show that contaminating Ca^{2+} is not coming from the measurement solution, the BAPTA solution, or the quartz cuvette, because calculation of $f_{background}$ yielded small and insignificant values. Calcium contamination in the TPEN solution also can be excluded because high $[Ca_T]_{WM}$ values were not restricted to the results after TPEN addition.

Based on the difference in BAPTA-measured Ca^{2+} levels determined before and after TPEN addition (see Eqs. 1 and 2, and Table 1), one may make a reasonable estimate of the concentration of total zinc ($[Zn_T]_{WM}$, mmole/kg) in biological samples. Table 2 lists the total zinc concentrations estimated for rat soleus and plantaris muscle tissues, which were calculated first as the difference in $[Ca_T]_{WM}$ with or without the addition of TPEN (simple subtraction; see Table 2). It is important to note that when using the simple subtraction method above to calculate total zinc concentration, the $\Delta\epsilon$ of Ca^{2+} /BAPTA binding ($\Delta\epsilon_{Ca}$) is used to calculate total zinc concentration from the fraction of absorbance shift caused by zinc (see Eqs. 3–5):

$$\frac{A_M - A_0}{\Delta\epsilon_{Ca} \cdot l} = [CaB]_M + [ZnB]_M + [CaB]_{background} + [ZnB]_{background} \quad (3)$$

Because contamination of both calcium and zinc in the measurement solution is negligible, Eq. 3 can be expressed as:

$$\frac{A_M - A_0}{\Delta\epsilon_{Ca} \cdot l} = [CaB]_M + [ZnB]_M, \quad (4)$$

or

$$\frac{A_M - A_0}{l} = \Delta\epsilon_{Ca} \cdot [CaB]_M + \Delta\epsilon_{Ca} \cdot [ZnB]_M. \quad (5)$$

A more accurate method to estimate total zinc would be to use the $\Delta\epsilon$ of Zn^{2+} /BAPTA binding ($\Delta\epsilon_{Zn}$) for the fraction of BAPTA absorbance shift caused by zinc, which can be expressed as the following:

$$\frac{A_M - A_0}{l} = \Delta\epsilon_{Ca} \cdot [CaB]_M + \Delta\epsilon_{Zn} \cdot [ZnB]_{M,corrected}. \quad (6)$$

By comparing Eq. 5 with 6, we obtain Eq. 7 below ($\Delta\epsilon_{Zn} = -3.98 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ and $\Delta\epsilon_{Ca} = -4.63 \text{ mM}^{-1} \cdot \text{cm}^{-1}$; obtained from Fig. 2):

$$[ZnB]_{M,corrected} = \frac{\Delta\epsilon_{Ca}}{\Delta\epsilon_{Zn}} \cdot [ZnB]_M. \quad (7)$$

It follows then that the normalized (normalizing for tissue wet weight and homogenate volume) total zinc concentration $[Zn_T]_{WM,corrected}$ should be calculated as:

$$[Zn_T]_{WM,corrected} = \frac{\Delta\epsilon_{Ca}}{\Delta\epsilon_{Zn}} \cdot [Zn_T]_{WM}. \quad (8)$$

$[Zn_T]_{WM}$ values after being corrected by this ratio are listed in Table 2 as $[Zn_T]_{WM,corrected}$. Because the difference between these extinction coefficients is small, the correction results in a small but significant difference between the estimated $[Zn_T]_{WM}$ and $[Zn_T]_{WM,corrected}$ values. Thus, we recommend always applying this correction to $[Zn_T]_{WM}$ values especially when they are larger relative to $[Ca_T]_{WM}$. A comparison of the estimates of the total zinc concentration using TPEN in combination with the BAPTA method (by simple subtraction: 0.32 ± 0.046 in soleus and 0.15 ± 0.031 in plantaris; by corrected simple subtraction: 0.37 ± 0.054 in soleus and 0.18 ± 0.036 in plantaris; mean \pm SEM; $n = 3$; mmole/kg) with those obtained by direct measurement using ICP-MS analysis (soleus, 0.53 ± 0.04 ; plantaris, 0.19 ± 0.01 ; mean \pm SEM; $n = 5$; mmole/kg; see Fig. 1) suggests that using TPEN in combination with the BAPTA method yields reasonable estimates of total zinc concentration. In addition, the data listed in Table 2 show that plantaris muscle had a significantly lower total zinc concentration when compared with soleus muscle. This result was confirmed by ICP-MS analysis (see Fig. 1).

Physiological significance of Zn^{2+} chelation by BAPTA in muscle tissues

The acetoxymethyl derivative of BAPTA (BAPTA-AM) is commonly used as a cell-permeable Ca^{2+} chelator to buffer cytosolic calcium transients and elucidate downstream Ca^{2+} -dependent signaling pathways. However, when conducting such experiments, one should be aware that cytosolic Zn^{2+} transients would be buffered as well. As an example, the chelation of both Ca^{2+} and Zn^{2+} by intracellular BAPTA can be estimated based on commonly accepted free cytosolic concentrations of Ca^{2+} and Zn^{2+} , as well as the dissociation constants of both Ca^{2+} and Zn^{2+} for BAPTA. Lamboley et al. (2015) reported the muscle cytosolic free Ca^{2+} to be 34 nM. The K_d of BAPTA for Ca^{2+} is reported as 110 nM (Tsien, 1980). The cytosolic free Zn^{2+} is widely recognized to be >100-fold less than the resting cytosolic free Ca^{2+} at 100 pM (Maret, 2015). Using 100 pM as the free cytosolic Zn^{2+} and 7.9 nM as the K_d of BAPTA for Zn^{2+} (Benters et al., 1997), and 10 μ M BAPTA as its intracellular concentration, only 0.47 nM Ca^{2+} and 0.10 pM Zn^{2+} will remain free; both ions are strongly chelated! Consider that Zn^{2+} sparks and Zn^{2+} transients have been visualized with Zn^{2+} -specific fluorophores in electrically stimulated cardiomyocytes (Tuncay et al., 2011), which were similar in time course to the known rapid changes in cytosolic calcium. In addition, treating cardiomyocytes with TPEN resulted in a significant decrease in the amplitude of Zn^{2+} transients. Tuncay et al. (2011) also provided evidence for possible intracellular pools responsible for the Zn^{2+} transients that included the SR, mitochondria, and Zn^{2+} -binding proteins. In addition, a recent finding suggests that Zn^{2+} can shape the kinetics of cardiac Ca^{2+} release by direct modulation of the type 2 ryanodine receptor (Woodier et al., 2015). As it can be seen here, if merely BAPTA-AM instead of TPEN/BAPTA-AM is used to study the above two processes, either the actual Zn^{2+} transient occurring in cardiac excitation-contraction cycle or the finely tuned Zn^{2+}/Ca^{2+} signaling might be missed. TPEN, being cell permeable, may be applied in parallel experiments to clarify if the observed effects of BAPTA are actually uncovering the actions of Zn^{2+} transients (Haase and Rink, 2014; Kočańczyk et al., 2015). However, researchers need to use caution when applying TPEN to living cells as it can be cytotoxic (Bozym et al., 2006), presumably because it is such an effective chelator of Zn^{2+} and other divalent metal ions.

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