# Chapter 16

# Calcium Uptake in Crude Tissue Preparation

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## Abstract

The various isoforms of the sarco/endoplasmic reticulum $Ca^{2+}$ ATPase (SERCA) are responsible for the $Ca^{2+}$ uptake from the cytosol into the endoplasmic or sarcoplasmic reticulum (ER/SR). In some tissues, the activity of SERCA can be modulated by binding partners, such as phospholamban and sarcolipin. The activity of SERCA can be characterized by its apparent affinity for $Ca^{2+}$ as well as maximal enzymatic velocity. Both parameters can be effectively determined by the protocol described here. Specifically, we describe the measurement of the rate of oxalate-facilitated $^{45}Ca$ uptake into the SR of crude mouse ventricular homogenates. This protocol can easily be adapted for different tissues and animal models as well as cultured cells.

**Key words** Calcium uptake, Calcium affinity, SERCA, Phospholamban, Sarcolipin, $^{45}Ca$, $K_m$, $V_{max}$, Oxalate

## 1 Introduction

The ability to mobilize $Ca^{2+}$ has been, for decades, a well-established characteristic of ER/SR microsomes [1–3]. This observation was of particular importance in muscle tissue where $Ca^{2+}$ is the trigger for contraction [4, 5]. This $Ca^{2+}$ uptake activity was later determined to be produced by an integral membrane ATPase protein, SERCA [6–8]. Further research identified phospholamban [9, 10], and later sarcolipin [11, 12], as inhibitors of SERCA activity in cardiac and skeletal muscles, respectively.

SERCA activity follows traditional Michaelis-Menten enzyme kinetics [13] described by the equation

$$v = \frac{V_{max}[S]}{K_m + [S]}$$

Here $v$ is the observed rate and $[S]$ is the concentration of rate-limiting substrate, which is $Ca^{2+}$ in this case. In other words, the rate of $Ca^{2+}$ binding to SERCA is the determinant of SERCA activity. (ATP is also a critical substrate for SERCA, but generally is well

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above saturating concentrations and therefore not rate limiting and not included in basic descriptions of SERCA activity). $V_{max}$ is the maximal rate of the enzyme and $K_m$ is the Michaelis constant which corresponds to the substrate concentration at the half maximal rate. $K_m$ is an inverse measure of substrate affinity, meaning that a low value corresponds to a high affinity and vice versa. In simpler terms — at low $Ca^{2+}$ concentrations, where little is available to be pumped across the membrane, SERCA activity is low. Elevating $Ca^{2+}$ increases SERCA activity up to a maximal level.

The $Ca^{2+}$ uptake protocol described here produces the Michaelis-Menten response curve of SERCA over the physiologically relevant $Ca^{2+}$ concentrations (10 nM to 10 μM), which also corresponds to the full dynamic range of the enzyme [10] (Fig. 1). For easy visualization and discussion, $Ca^{2+}$ concentrations are represented with pCa values, which correspond to the inverse log of the molar concentration.

Changes in PLN or SLN inhibition will generate a leftward or rightward shift in this activity curve signifying an altered $Ca^{2+}$ affinity. $V_{max}$ typically varies depending on SERCA expression level. Variations in $K_m$ and $V_{max}$ will also vary between species, tissue type, and SERCA isoform.

The protocol presented here is a detailed description of our standard laboratory procedure [14–20] and is adapted from the Millipore filtration technique [21]. In principle, this assay measures the amount of $^{45}Ca$ retained in homogenate microsomes over time after being transported by SERCA. These microsomes

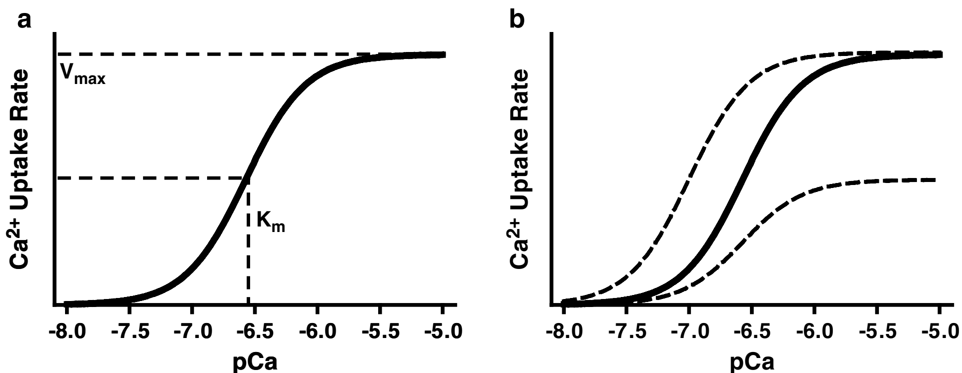


**Fig. 1** *Representative SERCA activity curves following Michaelis-Menten enzyme kinetics*. (**a**) $V_{max}$ describes the maximal uptake rate of the sample and $K_m$ indicates the $Ca^{2+}$ concentration at half the maximal rate. (**b**) The *dashed lines* showing downward and leftward shifts from the *solid black line* correspond to a decrease maximal rate ($V_{max}$) and an increase in $Ca^{2+}$ affinity ($K_m$), respectively. For cardiac preparations, a downward shift would correspond to the diminished SERCA protein expression as observed in heart failure. The leftward shift would indicate relief of PLN inhibition either through phosphorylation or ablation

are collected by a nitrocellulose membrane and subsequently washed to allow excess $Ca^{2+}$ that is not sequestered by the microsomes to pass through. Ruthenium Red blocks extrusion of $Ca^{2+}$ out of the microsomes through ion channels [22] and prevents uptake into the mitochondria [23]. $Ca^{2+}$ precipitates with oxalate inside ER/SR microsomes [24–26], which serves multiple purposes in this assay. First, this precipitation lowers the free $Ca^{2+}$ inside the microsomes, which eliminates the generation of a concentration gradient that would slow SERCA activity over time, thereby allowing consistent $Ca^{2+}$ transport for the duration of the assay [27, 28]. Secondly, it further prevents $Ca^{2+}$ extrusion out of the microsomes. Oxalate also preferentially accumulates in ER/SR microsomes via a nonspecific anion transporter [24–26, 29]. Therefore, the oxalate-trapped $Ca^{2+}$ resides in only ER/SR microsomes which eliminates the need for ER/SR purification that may introduce significant variability between samples.

It is important to note that this assay describes the initial rates of steady-state activity of SERCA [27], although the cytosolic environment is not at steady-state. Increased SERCA activity decreases cytosolic $Ca^{2+}$, thereby decreasing its own enzymatic activity.

## 2 Materials

### 2.1 Solutions

Prepare all stock solutions using ultrapure water and analytical grade reagents and store at 4 °C unless otherwise noted.

1. Homogenization Buffer: Prepare on the day of the experiment according to Table 1 and keep on ice until use.
2. Reaction Mixture: Prepare on the day of the experiment according to Table 2 and keep on ice until use.
3. 0.1 M ATP: For 75 mL, dissolve 4.27 g ATP (MW 569.1 g/mol) in 40 mL of $H_2O$ and adjust the pH to 7.0 using 1 N NaOH. Keep on ice. Bring the volume to 70 mL. Calculate the true concentration by measuring and averaging the absorbance at 259 nm of multiple dilutions (1:1000–1:4000). $M$ = Abs at 259 nm/$15.4 \times 10^3$. Dilute the sample to exactly 0.1 M, aliquot, and store at −80 °C.
4. $1.14 \times 10^{-4}$ M Ruthenium Red: The day of the experiment, dilute approximately 0.1 mg in 1 mL of water. Calculate the true concentration by measuring the absorbance at 533 nm at multiple dilutions (1:100–1:300). $M$ = Abs at 533 nm/$6.4 \times 10^4$. Dilute the sample to $1.14 \times 10^{-4}$ M. 400 μL are needed for each assay in duplicate.

**Table 1**
**Homogenization buffer**

| Stock | [Final] | Amount for 5 mL |
|---|---|---|
| 0.5 M phosphate buffer, pH 7.0 | 50 mM | 0.5 mL |
| 0.1 M NaF | 10 mM | 0.5 mL |
| 0.5 M EDTA | 1 mM | 0.01 mL |
| Sucrose | 0.3 M | 0.52 g |
| 0.02 M PMSF (–20 °C) | 0.3 mM | 0.075 mL |
| 0.1 M DTT (–20 °C) | 0.5 mM | 0.0125 mL |

**Table 2**
**Reaction mixture**

| Stock | [Final] (mM) | Amount for 30 reactions (mL) |
|---|---|---|
| 0.08 M Imidazol, pH 7.0 | 40 | 22.5 |
| 1 M KCl | 95 | 4.275 |
| 0.1 M $NaN_3$ | 5 | 2.25 |
| 0.1 M $MgCl_2$ | 5 | 2.25 |
| 0.5 M EGTA | 0.5 | 0.045 |
| 0.1 M $K^+$ oxalate | 5 | 2.25 |

5. $^{45}Ca$: Prepare an initial stock of $^{45}Ca$ to a concentration of 2.5 μCi/μL in $H_2O$. For each assay in duplicate, 900 μL of 40 μCi/mL (36 μCi) is needed. 36 μCi corresponds to 14.4 μL of a 2.5 μCi/μL stock. To account for decay, divide 14.4 μL by the decay factor obtained from a $^{45}Ca$ decay chart. Add $H_2O$ to bring final volume to 900 μL.
6. 10.00 mM $CaCl_2$: Either purchase an analytical grade calcium solution or have the concentration of a prepared stock analytically verified.
7. Wash Buffer: 20 mM Tris–HCl, 2 mM EGTA, pH 7.0.
8. Tissue of Interest: This assay is optimized for whole mouse ventricular cardiac tissue (approximately 20 mg) and can be adapted

for other tissue types or cultured cell lines. The abundance of SERCA protein, which is high in muscle, should be taken into account when adapting to non-muscle tissue or cells.

### 2.2 Equipment

1. Vacuum filtration system.
2. 0.45 μm nitrocellulose Millipore filters.
3. Water bath set to 37 °C.
4. Reaction tubes: 15 × 85 mm borosilicate glass culture tubes.
5. 20 mL scintillation vials.
6. Scintillation counter.
7. Tissue homogenizer.
8. Vortex.

## 3 Methods

### 3.1 Uptake Reaction

The key to this assay is consistent pipetting and great care should be taken to yield accurate and precise results. To further enhance accuracy, we recommend performing the entire assay in duplicate. Also, start at the lowest $Ca^{2+}$ concentration and move to higher ones. On the duplicate set of reactions, start at the highest $Ca^{2+}$ concentration and move to lower ones.

1. Set up the 13 reaction tubes in duplicate (26 total).
2. Set up 5 scintillation vials for each reaction: 1 blank, 1 standard, and 1 for each of the 3 time points (130 total).
3. Prepare reaction tubes according to Table 3.
4. Homogenize tissue in homogenization buffer and keep on ice until used for each reaction. 75 μL is necessary for each of the 26 reactions (1.950 mL total). Approximate protein concentration should be between 0.5 and 5.0 mg/mL. Final rates will be normalized to quantified concentration.
5. Place four 0.45 μm Millipore filters on filtration manifold for the first reaction tube.
6. Wash each filter two times with 2.5 mL Wash Buffer.
7. Add 13.2 μL of 0.114 mM Ruthenium Red (1 μM final) to the first reaction tube.
8. Add 75 μL of homogenates to the first reaction tube and slightly vortex (avoiding air bubble formation) to thoroughly mix.
9. Place the reaction tube in a 37 °C water bath.
10. After 30 s remove an aliquot of 290 μL for nonspecific binding (also called blank). Pass the aliquot through a 0.45 μm Millipore membrane.

**Table 3**
**Calcium dilutions**

| | | Volume to add (μL) | | | |
|---|---|---|---|---|---|
| Buffer # | pCa | 10.00 mM $CaCl_2$ | $dH_2O$ | $^{45}Ca$ (40 μCi/mL) | Reaction mixture |
| 1 | 8 | 2.6 | 228.3 | 1.9 | 1119 |
| 2 | 7.5 | 7.7 | 219.4 | 5.7 | 1119 |
| 3 | 7.2 | 13.9 | 208.7 | 10.2 | 1119 |
| 4 | 7 | 19.9 | 198.3 | 14.6 | 1119 |
| 5 | 6.8 | 27.4 | 185.2 | 20.2 | 1119 |
| 6 | 6.6 | 36.0 | 170.3 | 26.5 | 1119 |
| 7 | 6.4 | 44.8 | 155.0 | 33.0 | 1119 |
| 8 | 6.3 | 49.1 | 147.6 | 36.1 | 1119 |
| 9 | 6.2 | 53.2 | 140.5 | 39.1 | 1119 |
| 10 | 6 | 60.5 | 127.8 | 44.5 | 1119 |
| 11 | 5.8 | 66.8 | 116.9 | 49.1 | 1119 |
| 12 | 5.5 | 74.7 | 103.1 | 55.0 | 1119 |
| 13 | 5 | 89.9 | 76.8 | 66.1 | 1119 |

11. Initiate the reaction by adding 60 μL 0.1 M ATP, [Final] = 5 mM. Mix thoroughly with the pipet.
12. Take out 300 μL at 30, 60, and 90 s time points and pass each aliquot through a different membrane.
13. Wash each membrane two times with 2.5 mL wash buffer.
14. Place the filters in the scintillation vials.
15. Repeat **steps 5–14** for the remaining reaction tubes.
16. When finished, add 60 μL from the remaining reaction mixture to the standard scintillation vial for each reaction.
17. Add 10 mL of scintillation fluid to each scintillation vial.
18. Measure the samples with a scintillation counter.

### *3.2 Data Analysis*

1. Quantify homogenate protein concentration using the Bradford method.
2. The scintillation counter yields values in counts per minute (cpm) which can be converted to moles of $Ca^{2+}$ using the standard sample from **step 16** in Subheading 3.1. This sample has not been filtered and therefore is representative of the total

$Ca^{2+}$ in each reaction mixture. The amount of microsome sequestered $Ca^{2+}$ is represented by the following equation:

$$\text{Ca Uptake (nmol)} = \frac{\text{sample cpm} - \text{blank cpm}}{\text{standard cpm} \times 5} \times \text{Ca (nmol)}$$

Here Ca (nmol) represents the amount of cold $Ca^{2+}$ present in each aliquot (300 μL) of each individual reaction mixture (Table 3: 10.00 mM $CaCl_2$ column) which is one-fifth of the amount in the total reaction mixture (1.5 mL).

3. After all Ca-uptake values are calculated, perform a linear regression from the three time points (30, 60, and 90 s) for each pCa. The slope of this line corresponds to the rate of $Ca^{2+}$ uptake in nanomole/minute. These values should then be normalized to the amount of protein giving a final normalized rate in nmol/min/mg (Fig. 2a).
4. Plot the average of the duplicate calculations against the corresponding $Ca^{2+}$ concentration. This plot can then be fit with the Hill equation to determine the $V_{max}$ and $K_m$ parameters (Fig. 2b).

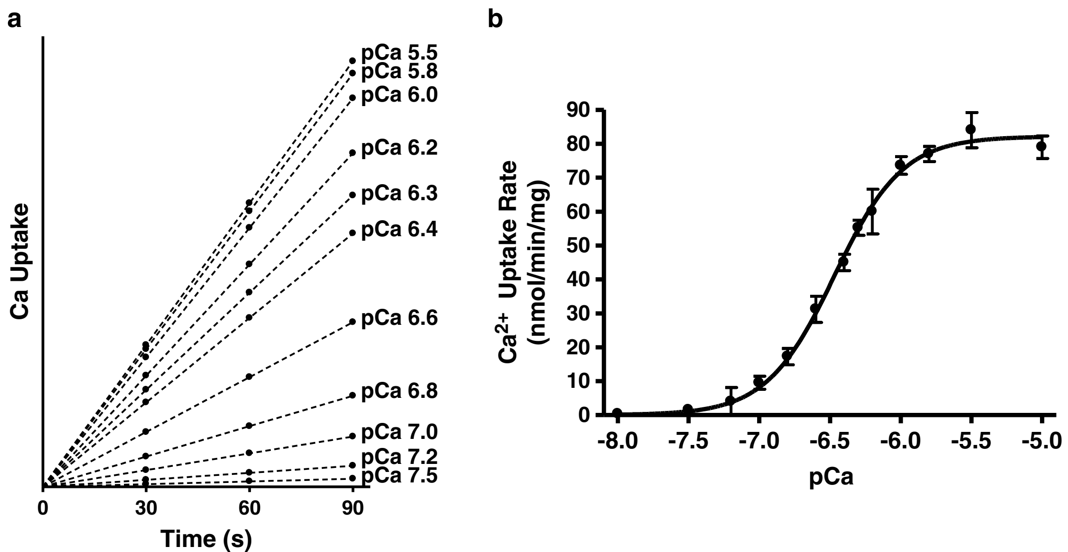


**Fig. 2** *Examples plots of the initial rates of SERCA activity from C57/BL6 mouse ventricular homogenates.* (**a**) The rate at each $Ca^{2+}$ concentration is determined by calculating the slope from the Ca-uptake values from the 3 time points. These rates can then be plotted against the $Ca^{2+}$ concentration (**b**) and fit with the Hill equation to determine the $V_{max}$ and $K_m$ parameters. For this example plot, $V_{max}$ = 80.2 ± 0.5 and $K_m$ = pCa 6.468 ± 0.007 (340 nM)

## 4 Notes

1. Be sure to follow the specific radiation safety guidelines at your facility for use and disposal of $^{45}Ca$.
2. For consistency between assays, use the same batch of stock solutions for all experiments if possible.
3. ATP and EGTA are critical for buffering $Mg^{2+}$ and $Ca^{2+}$ as changes in ATP concentration will cause variations in the free $Ca^{2+}$ concentration.
4. The stock $CaCl_2$ solution is perhaps the most critical element in this assay and is essential for accurate $K_m$ measurements.
5. Reaction tubes and scintillation vials can be organized and labeled the day prior to the assay.
6. To limit the time spent in the radioactive lab and overall exposure, $^{45}Ca$ should be added to the reaction mixture just prior to initiating the reaction.
7. The 30, 60, and 90 s time points correspond to when the sample is passed through the membrane. To ensure proper timing, start a timer when adding ATP. Remove an aliquot from the reaction tube in water bath 5–6 s before each time point for transport to filtration system. Then pipet the sample exactly at 30, 60, and 90 s.
8. Gently resuspend the reaction mixture before removing each aliquot to ensure even distribution of microsomes.
9. Washing can be facilitated by a bottle top repeat dispenser.
10. A 50× stock of wash buffer can be prepared and stored at 4 °C.
11. For consistency between individual assays performed over multiple days, freeze a small aliquot from each homogenate and perform protein quantification for all samples simultaneously.
12. Gently shake each scintillation vial to ensure that the membranes are fully submerged in scintillation fluid.
13. For ease of analysis, a template spreadsheet should be generated.
14. Calculated rates (slopes) with $R^2$ values less than 0.9 should be excluded from further analysis. These errors are likely due to inconsistent pipetting and/or inconsistent timing.
15. $K_m$ values are generally consistent and may only require a few repetitions to generate statistical significance. $V_{max}$ values are more inconsistent, though reliable values can be obtained by additional repetitions. If necessary, data can be normalized to $V_{max}$ values.

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