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Calcium Uptake in Crude Tissue Preparation

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SUMMARY

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.

Keywords

calcium uptake; calcium affinity; SERCA; phospholamban; sarcolipin; ^{45}Ca ; K_m ; V_{max} ; oxalate

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¹Be sure to follow the specific radiation safety guidelines at your facility for use and disposal of ^{45}Ca .

²For consistency between assays, use the same batch of stock solutions for all experiments if possible.

³ATP 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.

⁴The stock CaCl_2 solution is perhaps the most critical element in this assay and is essential for accurate K_m measurements.

⁵Reaction tubes and scintillation vials can be organized and labeled the day prior to the assay.

⁶To 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.

⁷The 30, 60, and 90 second 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 seconds before each time point for transport to filtration system. Then pipet the sample exactly at 30, 60, and 90 seconds.

⁸Gently resuspend the reaction mixture before removing each aliquot to ensure even distribution of microsomes.

⁹Washing can be facilitated by a bottle top repeat dispenser.

¹⁰A 50x stock of Wash Buffer can be prepared and stored at 4°C.

¹¹For consistency between individual assays performed over multiple days, freeze a small aliquot from each homogenate and perform protein quantification for all samples simultaneously.

¹²Gently shake each scintillation vial to ensure that the membranes are fully submerged in scintillation fluid.

¹³For ease of analysis, a template spreadsheet should be generated.

¹⁴Calculated 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.

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 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 Michealis 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] (Figure 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 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 non-specific 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 to 1:4000). $M = \text{Abs at } 259 \text{ nm} / 15.4 \times 10^3$. Dilute the sample to exactly 0.1 M, aliquot, and store at -80°C .
4. **1.14×10^{-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 to 1:300). $M = \text{Abs at } 533 \text{ nm} / 6.4 \times 10^4$. Dilute the sample to 1.14×10^{-4} M. 400 μl are needed for each assay in duplicate.
5. **^{45}Ca :** Prepare an initial stock of ^{45}Ca to a concentration of 2.5 $\mu\text{Ci}/\mu\text{l}$ in H_2O . For each assay in duplicate, 900 μl of 40 $\mu\text{Ci}/\text{ml}$ (36 μCi) is needed. 36 μCi corresponds to 14.4 μl of a 2.5 $\mu\text{Ci}/\mu\text{l}$ 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 μ m 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 μ l 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 μ m Millipore filters on filtration manifold for the first reaction tube.
6. Wash each filter 2 times with 2.5 ml Wash Buffer.
7. Add 13.2 μ l of 0.114 mM Ruthenium Red (1 μ M final) to first reaction tube.
8. Add 75 μ l 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 seconds remove an aliquot of 290 μ l for nonspecific binding (also called blank). Pass the aliquot through a 0.45 μ m Millipore membrane.
11. Initiate the reaction by adding 60 μ l 0.1 M ATP, [Final] = 5 mM. Mix thoroughly with the pipet.
12. Take out 300 μ l at 30, 60, and 90 second time points and pass each aliquot through a different membrane.

13. Wash each membrane 2 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 μ l 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 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 section 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

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

Here $Ca \text{ (nmol)}$ represents the amount of cold Ca^{2+} present in each aliquot (300 μ l) 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 3 times points (30, 60, and 90 seconds) for each pCa. The slope of this line corresponds to the rate of Ca^{2+} uptake in nmol/min. These values should then be normalized to the amount of protein giving a final normalized rate in nmol/min/mg (Figure 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 (Figure 2B).

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References

1. Ebashi S, Lipmann F. Adenosine triphosphate-linked concentration of calcium ions in a particulate fraction of rabbit muscle. *J Cell Biol.* 1962; 14:389–400. [PubMed: 19866605]
2. Hasselbach W, Makinose M. The calcium pump of the “relaxing granules” of muscle and its dependence on ATP-splitting. *Biochem Z.* 1962; 333:518–28. [PubMed: 13712164]
3. Hasselbach W, Makinose M. On the Mechanism of Calcium Transport Across the Membrane of the Sarcoplasmic Reticulum. *Biochem Z.* 1963; 339:94–111. [PubMed: 14095160]

4. Gordon AM, Homsher E, Regnier M. Regulation of contraction in striated muscle. *Physiol Rev*. 2000; 80:853–924. [PubMed: 10747208]
5. Bers DM. Calcium cycling and signaling in cardiac myocytes. *Annu Rev Physiol*. 2008; 70:23–49. [PubMed: 17988210]
6. MacLennan DH, Brandl CJ, Korczak B, Green NM. Amino-acid sequence of a Ca^{2+} + Mg^{2+} -dependent ATPase from rabbit muscle sarcoplasmic reticulum, deduced from its complementary DNA sequence. *Nature*. 1985; 316:696–700. [PubMed: 2993904]
7. Brandl CJ, Green NM, Korczak B, MacLennan DH. Two Ca^{2+} ATPase genes: homologies and mechanistic implications of deduced amino acid sequences. *Cell*. 1986; 44:597–607. [PubMed: 2936465]
8. MacLennan DH. Purification and properties of an adenosine triphosphatase from sarcoplasmic reticulum. *J Biol Chem*. 1970; 245:4508–18. [PubMed: 4250726]
9. Tada M, Kirchberger MA, Katz AM. Phosphorylation of a 22,000-dalton component of the cardiac sarcoplasmic reticulum by adenosine 3':5'-monophosphate-dependent protein kinase. *J Biol Chem*. 1975; 250:2640–7. [PubMed: 235523]
10. MacLennan DH, Kranias EG. Phospholamban: a crucial regulator of cardiac contractility. *Nat Rev Mol Cell Biol*. 2003; 4:566–77.10.1038/nrm1151 [PubMed: 12838339]
11. Odermatt A, Becker S, Khanna VK, et al. Sarcolipin regulates the activity of SERCA1, the fast-twitch skeletal muscle sarcoplasmic reticulum Ca^{2+} -ATPase. *J Biol Chem*. 1998; 273:12360–9. [PubMed: 9575189]
12. MacLennan DH, Asahi M, Tupling AR. The regulation of SERCA-type pumps by phospholamban and sarcolipin. *Ann N Y Acad Sci*. 2003; 986:472–80. [PubMed: 12763867]
13. Inesi G, Kurzmack M, Coan CR, Lewis DE. Cooperative calcium binding and ATPase activation in sarcoplasmic reticulum vesicles. *J Biol Chem*. 1980; 255:3025–31. [PubMed: 6244305]
14. Haghighi K, Pritchard T, Bossuyt J, et al. The human phospholamban Arg14-deletion mutant localizes to plasma membrane and interacts with the Na/K-ATPase. *J Mol Cell Cardiol*. 2012; 52:773–82.10.1016/j.yjmcc.2011.11.012 [PubMed: 22155237]
15. Schmidt AG, Zhai J, Carr AN, et al. Structural and functional implications of the phospholamban hinge domain: impaired SR Ca^{2+} uptake as a primary cause of heart failure. *Cardiovasc Res*. 2002; 56:248–59. [PubMed: 12393095]
16. Frank K, Tilgmann C, Shannon TR, et al. Regulatory role of phospholamban in the efficiency of cardiac sarcoplasmic reticulum Ca^{2+} transport. *Biochemistry*. 2000; 39:14176–82. [PubMed: 11087366]
17. Zhai J, Schmidt AG, Hoit BD, et al. Cardiac-specific overexpression of a superinhibitory pentameric phospholamban mutant enhances inhibition of cardiac function in vivo. *J Biol Chem*. 2000; 275:10538–44. [PubMed: 10744747]
18. Harrer JM, Haghighi K, Kim HW, et al. Coordinate regulation of SR Ca^{2+} -ATPase and phospholamban expression in developing murine heart. *Am J Physiol*. 1997; 272:H57–66. [PubMed: 9038922]
19. Szymanska G, Grupp IL, Slack JP, et al. Alterations in sarcoplasmic reticulum calcium uptake, relaxation parameters and their responses to beta-adrenergic agonists in the developing rabbit heart. *J Mol Cell Cardiol*. 1995; 27:1819–29. [PubMed: 8523443]
20. Zhao W, Waggoner JR, Zhang Z-G, et al. The anti-apoptotic protein HAX-1 is a regulator of cardiac function. *Proc Natl Acad Sci U S A*. 2009; 106:20776–81. [PubMed: 19920172]
21. Solaro RJ, Briggs FN. Estimating the Functional Capabilities of Sarcoplasmic Reticulum in Cardiac Muscle. *Circ Res*. 1974; 34:531–540. [PubMed: 4826929]
22. Chamberlain BK, Volpe P, Fleischer S. Inhibition of calcium-induced calcium release from purified cardiac sarcoplasmic reticulum vesicles. *J Biol Chem*. 1984; 259:7547–53. [PubMed: 6736019]
23. Moore CL. Specific inhibition of mitochondrial Ca^{++} transport by ruthenium red. *Biochem Biophys Res Commun*. 1971; 42:298–305. [PubMed: 4250976]
24. Beil FU, von Chak D, Hasselbach W, Weber HH. Competition between oxalate and phosphate during active calcium accumulation by sarcoplasmic vesicles. *Z Naturforsch C*. 1977; 32:281–7. [PubMed: 141806]

25. De Meis L, Hasselbach W, Machado RD. Characterization of calcium oxalate and calcium phosphate deposits in sarcoplasmic reticulum vesicles. *J Cell Biol.* 1974; 62:505–9. [PubMed: 4139164]
26. Feher JJ, Lipford GB. Calcium oxalate and calcium phosphate capacities of cardiac sarcoplasmic reticulum. *Biochim Biophys Acta.* 1985; 818:373–85. [PubMed: 3876113]
27. Madeira VM. State of translocated Ca^{2+} by sarcoplasmic reticulum inferred from kinetic analysis of calcium oxalate precipitation. *Biochim Biophys Acta.* 1984; 769:284–90. [PubMed: 6230104]
28. Inesi G, de Meis L. Regulation of steady state filling in sarcoplasmic reticulum. Roles of back-inhibition, leakage, and slippage of the calcium pump. *J Biol Chem.* 1989; 264:5929–36. [PubMed: 2522442]
29. MacLennan DH, Reithmeier RA, Shoshan V, et al. Ion pathways in proteins of the sarcoplasmic reticulum. *Ann N Y Acad Sci.* 1980; 358:138–48. [PubMed: 6259987]

Figure 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 Ca²⁺ 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 Ca²⁺ 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.

Figure 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, $V_{max} = 80.2 \pm 0.5$ and $K_m = \text{pCa } 6.468 \pm 0.007$ (340 nM).

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]	Amount for 30 Reactions
0.08 M Imidazol, pH 7.0	40 mM	22.5 ml
1 M KCl	95 mM	4.275 ml
0.1 M NaN ₃	5 mM	2.25 ml
0.1 M MgCl ₂	5 mM	2.25 ml
0.5 M EGTA	0.5 mM	0.045 ml
0.1 M K ⁺ oxalate	5 mM	2.25 ml

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Table 3

Calcium Dilutions

Buffer #	pCa	10.00 mM CaCl ₂	Volume to Add (μl)		
			dH ₂ O	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