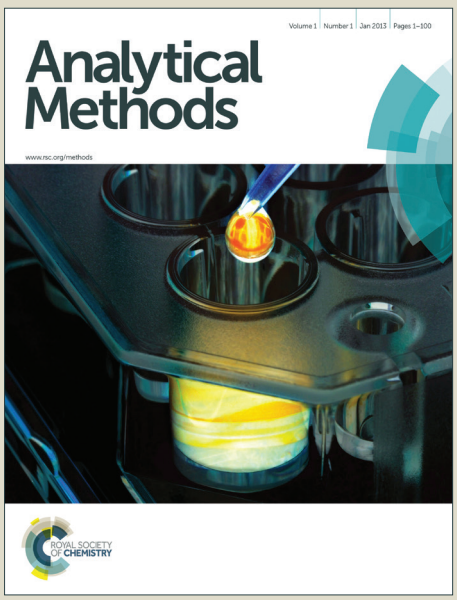
Analytical Methods

Accepted Manuscript



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Development of a Sensitive Assay for SERCA Activity Using FRET Detection of ADP

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Abstract

Various isoforms of sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA) regulate Ca^{2+} homeostatic balance in both the heart (SERCA2a) and skeletal muscle (SERCA1a). Ca^{2+} plays a key role in these tissues as an intracellular signal that controls contractility. Due to its key role in the contractility cycle, SERCA is emerging as a promising pharmacological target to modulate heart muscle function. SERCA function is regulated by its endogenous inhibitor phospholamban (PLN). Upon binding, PLN decreases SERCA's apparent affinity for Ca^{2+}. Therefore the interaction between PLN and SERCA has an important role in determining both physiological and pathological conditions. Quantifying the inhibitory potency of PLN is of great importance in understanding the pathophysiology of heart muscle. Traditionally, SERCA activity assays have been performed using a PK/LDH-coupled enzyme reaction, which suffers from limited sensitivity. We have developed a new SERCA activity assay based on the direct detection of the product ADP via time resolved FRET (TR-FRET). Under optimized conditions, our assay reduced the amount of SERCA required to perform the assay 1,000-fold. Inter-day reproducibility was shown to be excellent for SERCA preparations in either detergent ($C_{12}E_8$) or reconstituted lipids. The inhibitory effect of PLN on SERCA measured under the low-concentration conditions of our assay allowed us to more accurately investigate the binding between PLN and SERCA. Significant inhibitory effects of PLN were observed even at mid-nanomolar concentrations significantly lower than previous K_d estimates for the SERCA-PLN complex.

Introduction

The sarco(endo)plasmic reticulum Ca^{2+} ATPase (SERCA) is a membrane protein that translocates Ca^{2+} ions from the cytosol to the lumen, initiating cardiac muscle relaxation.[1-4] Phospholamban (PLN), a single-pass membrane protein, co-localizes with SERCA in the cardiac sarcoplasmic reticulum,[5] interacting with the ATPase in both cytoplasmic[6] and transmembrane[7] domains and regulating Ca^{2+} flux.[8] Ca^{2+} translocation in the sarcoplasmic reticulum by SERCA is driven by ATP hydrolysis. For each ATP molecule hydrolyzed to ADP two Ca^{2+} ions are transported through the transmembrane domains. Muscle contractility strongly depends on the Ca^{2+} homeostatic balance and SERCA function. Therefore, quantification of SERCA activity and its down-regulation by PLN are

fundamental requirements for understanding muscle physiology and pathophysiology as well providing an opportunity for the development of new drugs to counteract heart muscle dysfunctions via this regulatory pathway.[9]

Although SERCA as an ATPase, it does not produce a phosphorylated substrate,[10-13] suggesting that assays that directly monitor the consumption of ATP or production of ADP would be desirable for developing an assay for SERCA activity. One approach is to quantify ATP consumption using a chemiluminescent assay, in which luciferin and luciferase are added after a predetermined reaction time to consume the residual ATP and generate light.[14, 15] A major drawback of this method is that at least 20% to 30% ATP consumption is necessary to reach the desired detection window.[16] However, from the Michaelis-Menten equation V_{max} is only obtained when the concentration of ATP is much higher than K_m, and therefore ATP consumption is too little to be determined accurately using this method.[17] Other reported approaches for monitoring SERCA activity include monitoring production of pyrophosphate[18] or translocation of $^{45}Ca^{2+}$ into microsomes.[19]

Alternatively, ADP production generated by the ATPase can be detected to measure the enzyme's activity. Traditionally, a coupled enzyme assay involving a pyruvate kinase (PK) and lactate dehydrogenase (LDH) has been used to detect ADP in a continuous mode.[20-25] Phosphoenolpyruvic acid (PEP) is first converted to pyruvate, which is then hydrogenated to form lactate, and which is coupled with the dehydrogenation of NADH to NAD^+. The decrease of NADH is monitored using UV-Vis absorbance at 340 nm to estimate the rate of the enzyme reaction. Although widely applied in kinase and ATPase assays, this method has several disadvantages. For example, the detection is based on UV, which has relatively low sensitivity; the method requires large amounts of both enzyme and ATP; and sometimes it suffers from emission overlap between NADH and other reagents or NADH decomposition.[26] Other assays have been developed to overcome these drawbacks. For example, an improved coupled enzyme reaction used PK, pyruvate oxidase, and horseradish peroxidase to detect ADP via fluorescence, which avoided UV detection interference.[16] In an alternative approach, a dithio-coupled kinase or ATPase assay was developed with a ATPβS (sulfur on the β-phosphorous) substrate replacing ATP. Dithiol reagents such as DSSA and DTNB were used to react with the ADPβS product, and absorbance or fluorescence was detected in a continuous mode.[27] Assay sensitivity was improved by using fluorescence or reagents with higher extinction coefficients. Unfortunately, the labeled substrate ATPβS is not commercially available. Moreover, all these assays targeting ADP production employed multiple reagents and coupled reactions. These indirect detection methods raise the possibility of nonspecific interactions between inhibitor/screening drug and detection mixtures. In addition, assay validation is

necessary in order to report enzyme reaction rate accurately and confirm that the coupled enzyme reactions or dithio labeling reactions are not rate-limiting.

In this manuscript, we report the development of a highly sensitive SERCA activity assay using TR-FRET detection. This immunoassay is based on the Transcreener® platform, whose key component, an ADP specific antibody, directly binds the product of the SERCA reaction, eliminating coupled enzyme reactions or extra labeling reactions.[28] The far red TR-FRET detection avoids interfering fluorescence or light scattering from the reaction mixture. The homogenous assay was performed in 384-well plate with only 20 µL total volume in each reaction reducing the amount of SERCA required by three orders of magnitude. Under the optimized conditions, SERCA activity assays performed in both detergent ($C_{12}E_8$) and reconstituted lipid (DOPC: DOPE, 4:1) preparations showed good reproducibility.

Experimental Methods

Materials and Chemicals

All chemicals were purchased from Sigma Aldrich (St. Louis, MO) at the highest grade available, except 3-morpholinopropanesulfonic acid (MOPS, 99.5%, Fluka Analytical), $CaCl_2$ (99.9%, Mallinckrodt Baker), $MgCl_2$ (99.8%, Mallinckrodt Baker), KCl (99.5%, Mallinckrodt Baker), and NaCl (99.0%, Spectrum). All buffers were prepared in deionized water from a Milli-Q water purification system (Millipore Corp., Bedford, MA). Transcreener® ADP^2 Assay TR-FRET Red kit was purchased from BellBrook Labs (Madison, WI). SERCA1a was extracted and purified from rabbit skeletal muscle and co-reconstituted in lipid membranes (dioleoyl phosphatidylcholine: dioleoyl phosphatidylethanolamine, 4:1, or DOPC: DOPE, 4:1). This sample consisted of 8 µg SERCA, 22 µg PLN, 0.2 mg lipids (DOPC: DOPE, 4:1), 35 mM imidozole, 100 mM KCl, 5 mM $MgCl_2$, and 10% (v/v) glycerol at pH 7.0 in a final volume of 200 µL, which gave a SERCA concentration of 364 nM and a PLN concentration of 18.3 µM (calculated based on the molecular weight of the PLN monomer). Another two samples were reconstituted with 80 µg SERCA, and 80 µg of SERCA and 22 µg of PLN, respectively, following the same preparation procedure. 2×assay buffer 1 composed of 100 mM MOPS, 200 mM KCl, 10 mM $MgCl_2$, and 2 mM EGTA at pH 7.0 (pH adjusted by 1 M NaOH). Assay buffer 2 composed of 20 mM MOPS, 1 mM $MgCl_2$, 0.25 mM DTT, 0.1% (w/v) Octaethylene glycol monododecyl ether ($C_{12}E_8$), and 40% (v/v) glycerol at pH 7.2 (pH adjusted by 1 M NaOH). Assay buffer 3 composed of 1 µg/µL lipids (DOPC: DOPE, 4:1), 35 mM Imidozole, 100 mM KCl, 5 mM $MgCl_2$, and 10% (v/v) glycerol at pH 7.0.

SERCA Extraction and Purification

SERCA preparation was achieved following the protocol of Stokes and co-workers.[29] Briefly, an initial fractionation of the rabbit skeletal muscle tissue was carried out by homogenizing fresh muscle preparations followed by centrifugation to remove unwanted tissues. Subsequently, high speed centrifugation was used to remove excess cellular components, such as mitochondria, to isolate lipids and proteins of the sarcoplasmic reticulum (SR).[30] The sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA) accounts for approximately 50-80% of the total protein of the SR and was further purified using a sucrose gradient centrifugation step followed by affinity chromatography.[29, 31] In order to obtain functional SERCA from the SR preparation, the sample was treated with octaethylene glycol dodecylether (C12E8).[21, 25] After another centrifugation step, the solubilized SERCA was loaded onto a reactive-red affinity column which binds SERCA selectively. After several washing steps, SERCA was eluted from the column using ADP in the buffer. SERCA concentration was determined using a Lowry Assay (6).[32]

Instrument Settings

TR-FRET measurements were performed on a Synergy™ 2 Microplate Reader (BioTek Instruments, Inc., Winooski, VT) with filter settings of λ_{ex} = 360 ± 20 nm, λ_{em1} = 620 ± 5 nm, and λ_{em2} = 665 ± 4 nm. A 400 nm dichroic mirror was used with an excitation range of 320-390 nm and an emission range of 410-800 nm. Detection sensitivity was set to 135. A 20 sec shaking step was performed before taking any readings to eliminate possible bubbles in sample wells. Xenon flash with high lamp energy was used as the light source. There was a 100 ms delay after plate movement and before taking measurements. 100 µs after the xenon flash the instrument started data collection for 200 µs. Each reported FRET value was an average of 40 measurements.

Optimizing the Assay Detection Mixture

Since SERCA activity assays are performed in reaction buffers containing detergents or lipids, assay detection components including ATP and ADP tracer were first titrated to identify the best working concentrations. For tracer titration, 20 µL mixtures composed of 4 nM antibody, 2 µM ADP or ATP, 10 mM EDTA, and a series of ADP tracer ranging from 0.001 nM to 1 µM were incubated in assay buffer 2 for 1 h before being loaded into a Corning® 384 plate (catalog #3673) for TR-FRET measurements. The same experiments were also performed at 10 µM and 20 µM ADP or ATP concentrations. After the best ADP tracer concentration was determined, ATP titration was performed with 20 µL mixtures consisting of 4 nM antibody, ADP tracer at optimized concentration, and a series of ATP and ADP ranging from 0.01 µM to 1 mM.

Calibration Curve of ATP to ADP Conversion

A calibration curve of ATP to ADP conversion was recorded prior to each SERCA activity assay, in order to translate the FRET signal into the amount of ATP consumed or ADP produced in the reaction. 10 µL of ATP prepared in the desired assay buffer at twice the assay concentration was aliquoted into 20 vials. 20 µL of ADP at the same concentration was mixed with the 1st vial of ATP until even; 20 µL from the 1st vial was then transferred into the 2nd vial of ATP and mixed until even. The same procedure was performed until the last one. 10 µL of ATP-ADP mixtures were then incubated with 10 µL of the assay detection mixture at twice the optimized concentration. The final 20 µL mixtures were incubated at room temperature for 1 h before taking FRET measurements. The calibration curve was obtained based on three parallel experiments.

SERCA Titration

For SERCA titrations performed in $C_{12}E_8$, serial dilutions of SERCA were prepared in assay buffer 2 with the 39 µM stock SERCA originally in 20 mM MOPS, 1 mM $MgCl_2$, 0.25 mM DTT, 0.1% (w/v) $C_{12}E_8$, 30% (v/v) glycerol, and 200 µM $CaCl_2$. 5 µL aliquots of 8 µM ATP and 10 µM $CaCl_2$ prepared in assay buffer 2 were added into the same volume of SERCA serial dilutions to start the reactions at 37 °C in a C24 Incubator Shaker (New Brunswick Scientific, Enfield, CT). The reactions were quenched 5 min or 18 min later by adding 10 µL aliquots of 2× assay detection mixture containing excess EDTA to reach a final volume of 20 µL. For SERCA activity assays performed in reconstituted lipids, serial dilutions of SERCA were prepared in assay buffer 3. 5 µL aliquots of 8 µM ATP, 2.5 mM $CaCl_2$, and 7 µg/mL Ca^{2+} ionophore A23187 prepared in 2×assay buffer 1 were added to the same volume of SERCA serial dilutions to initiate the reactions. Reactions were quenched by the addition of 10 µL aliquots of 2× assay detection mixture.

ATPase Activity Measurements

For activity assays performed in $C_{12}E_8$, 5 µL aliquots of 8 µM ATP and varying concentrations of $CaCl_2$ were prepared in assay buffer 2. SERCA at twice the desired working concentration was also prepared in assay buffer 2. Reactions were started by adding 5 µL of SERCA into 5 µL of ATP-$CaCl_2$ mixture, and quenched by adding 10 µL of 2× assay detection mixture containing excess EDTA. For every Ca^{2+} concentration, reactions were quenched at five different reaction time points, and three parallel experiments were performed for each reaction time. Initial reaction rates at different Ca^{2+} concentrations were determined from the linear region of the FRET versus reaction time plots. Activity assays were performed similarly for SERCA in reconstituted lipids, except that 8 µM ATP, 7

μg/mL Ca^{2+} ionophore A23187, and varying concentrations of $CaCl_2$ were prepared in 2×assay buffer 1; SERCA at twice the desired working concentration was prepared in assay buffer 3. All reactions took place at 37 °C. Initial reaction rates at different Ca^{2+} concentrations were then fitted to the Hill equation to estimate pK_{Ca} values:

$$V = \frac{V_{max}}{1+10^{-n(pK_{Ca}-pCa)}} \quad (1)$$

where V is the initial reaction rate, V_{max} is the maximum initial reaction rate, n is the Hill coefficient, and pK_{Ca} is the pCa value when the initial reaction rate is at half V_{max}.

Results and Discussion

Assay Principle

The detection principle of the assay is shown in Figure 1. The Terbium labeled ADP antibody selectively binds HiLyte647 labeled ADP tracer in the presence of ATP, with an approximate 100-fold selectivity for ADP over ATP.[33] The binding between ADP antibody and ADP tracer brings the Terbium dye and HiLyte647 dye in close proximity, allowing Förster Resonance Energy Transfer (FRET) to take place. Emission was recorded at both 665 nm and 620 nm. When SERCA reacts with ATP, the ADP molecules produced compete with the HiLyte647 labeled ADP tracer for binding sites on the ADP antibody. The more ADP produced, the more ADP tracer is displaced, resulting in a smaller FRET emission signal at 665 nm. Detection was time resolved, collecting emission for a period of 200 μs starting 100 μs after the application of the excitory xenon flash, eliminating transient fluorescence interference of from other molecules. Detecting emission at wavelengths longer than 600 nm also mediates the effects of light scattering.

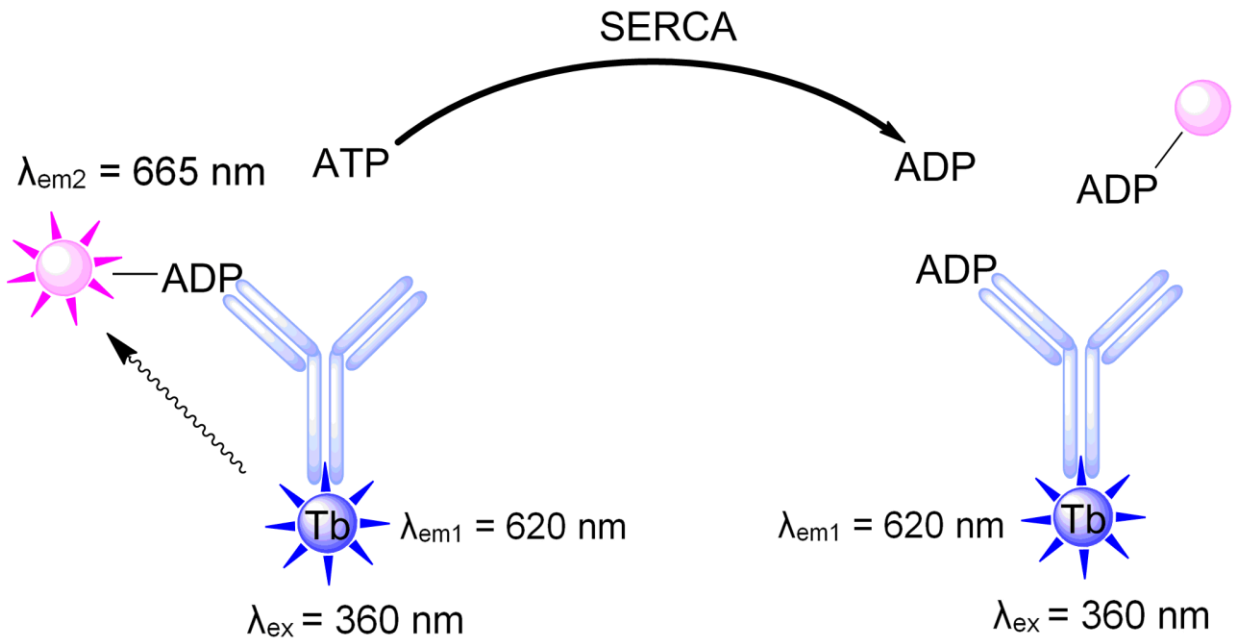

Figure 1. Schematic of the SERCA activity assay based on TR-FRET detection of ADP.

Optimizing Tracer and Antibody Concentration to Set the Detection Window

In order to obtain the SERCA activity curve, it is necessary to perform SERCA reactions at different Ca^{2+} concentrations, quench the reactions at the appropriate time, quantify the ADP produced, and finally obtain the initial reaction rates using a linear regression. To obtain initial reaction rates, it is necessary to quench reactions while substantial amounts of ATP still remain in the reaction mixture. Therefore it is necessary to ensure accurate ADP quantification in the presence of excess ATP. This selectivity requires optimization of the detection window to achieve large changes in the FRET signal even when ATP to ADP conversion is low.

Initially, tracer concentrations were set according to the linear relationship with starting ATP concentration as described in the TRANSCREENER® ADP^2 TR-FRET Red Assay Technical Manual. However, the Z'-factors, which are defined by the means and standard deviations of FRET signals of six replicates of ATP and ADP samples (see equation 2), obtained under these conditions were below the required specifications (<0.7, data not shown). It was therefore necessary to perform a tracer titration in our own working buffer to maximize the dynamic range of the assay.

$$Z' = 1 - \frac{3 \times SD_{ATP} + 3 \times SD_{ADP}}{\overline{FRET_{ATP}} - \overline{FRET_{ADP}}} \quad (2)$$

Figure 2 shows the tracer titration performed in assay buffer 2 containing the nonionic detergent $C_{12}E_8$. The red circles in the left panel indicate the FRET signals when 2 μM, 10 μM, and 20 μM ATP were incubated with different amounts of tracer, and the black squares indicate FRET signals when the same amounts of ADP were incubated with tracer. Since the antibody has much higher binding affinity for ADP over ATP, more ADP tracer molecules were displaced by ADP rather than ATP when tracer concentrations are within a certain range (e.g., 1 nM to 1 μM in Figure 2 (A)). When tracer concentration was below this range, both ATP and ADP were in excess to compete for binding sites, so that the FRET signals fell to baseline; when above this range, the tracer was in excess and neither ATP nor ADP would trigger a significant decrease of FRET signal. By subtracting the ADP FRET signals from the ATP FRET signals for all tracer concentrations, the FRET signal differences were obtained. From the right panel, the maximum FRET signal differences occur at ~ 63 nM, 250 nM, and 500 nM for 2 μM, 10 μM, and 20 μM adenosine, respectively. Using these tracer concentrations, the Z' factors were consistently above 0.7 from day-to-day experiments, indicating that a sufficient change in FRET signal was achieved under these conditions.

The right panel in Figure 2 clearly shows the tracer concentrations where the assay is optimized to best distinguish ATP from ADP. Similarly, the maximum FRET differences also represent the maximum assay detection window, since the ATP curve recreates the initial reaction conditions while the ADP curve recreates the endpoint of the reaction where all of the ATP has been consumed. The maximum assay window was approximately 0.120, 0.120, and 0.135 for assays starting with 4 μM, 20 μM, and 40 μM ATP (the addition of detection mixture dilutes the adenosine concentration in half). Although the assay window slightly decreased from 20 μM ATP and 500 nM tracer to 2 μM ATP and 63 nM tracer, the latter conditions reduced the amount of tracer required 8-fold, and the Z' value remained above 0.7. Most importantly, lowering the reagent concentrations required less SERCA to convert ATP to ADP in the same time, resulting in an overall improvement in assay sensitivity. Furthermore, titrations of ATP and ADP were performed with 63 nM tracer and 4 nM antibody, as shown in Figure 3 (A). The maximum FRET signal difference occured at ~2 μM adenosine (Figure 3 (B)), showing good self-consistency of the detection mixture.

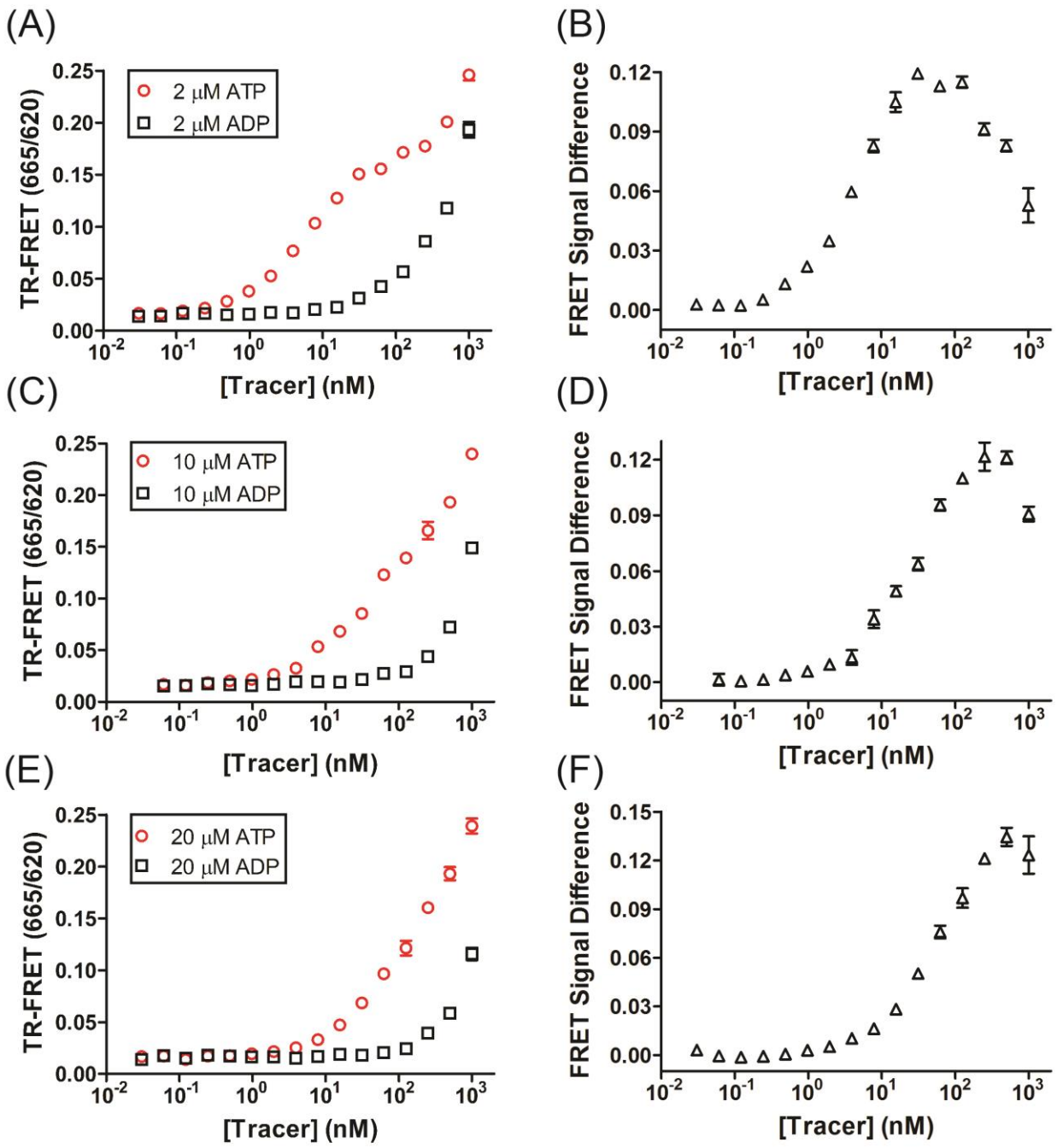

Figure 2. Tracer titration in assay buffer 2 with different concentrations of ATP and ADP: (A) 2 μM, (C) 10 μM, (E) 20 μM, using 4 nM ADP antibody. FRET signal differences between ATP and ADP at different tracer concentrations were shown in the right panel, with (B), (D), and (F) corresponding to (A), (C), and (E), respectively. Each point is the average FRET value of three replicate samples. Error bars represent standard deviation.

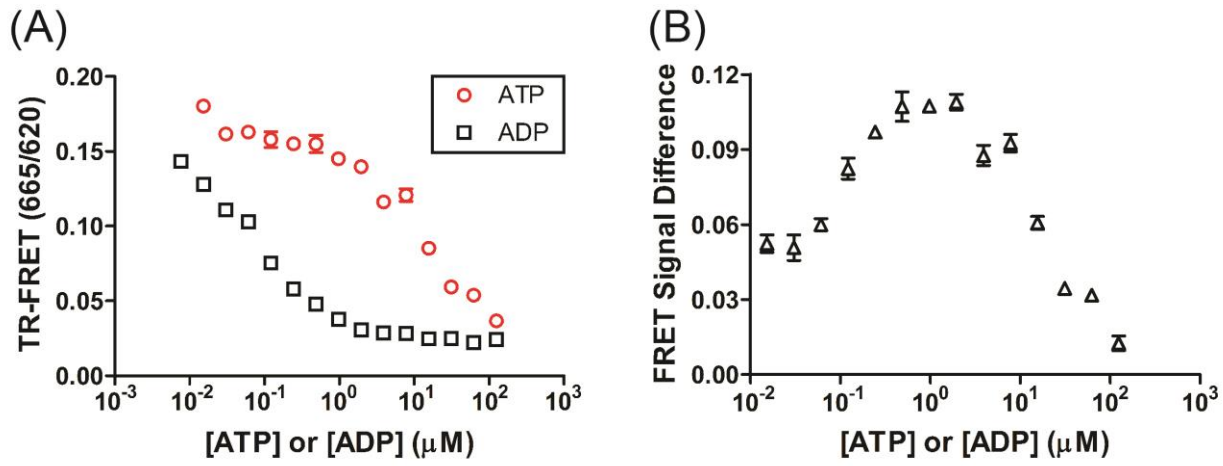

Figure 3. (A) ATP and ADP titrations in assay buffer 2 with 63 nM tracer and 4 nM ADP antibody. (B) FRET signal differences between ATP and ADP under the same adenosine concentrations. Each point is the average FRET value of three parallel samples. Error bars represent standard deviation.

Standard Calibration Curve

After optimization of the assay detection mixture, the standard calibration curve was obtained for SERCA reactions starting with 4 μM ATP. Figure 4 (A) shows the FRET values of ATP and ADP mixtures, which had a total adenosine concentration of 2 μM (the addition of detection mixture dilutes the adenosine concentration to half) but different compositions of ATP and ADP, simulating SERCA reactions taking place for different reaction times. As shown in Figure 4 (A), although no significant signal change occurred when ATP to ADP conversion was within 1%, FRET signal was linearly correlated to the conversion in the range of 1% to 100% in this semilog plot. The linear portion gave a regression equation of $y= -(0.051 \pm 0.001) \times \log(x) + (0.140 \pm 0.001)$, with R^2 of 0.9861. It is clear from this calibration curve that 50% of the maximum FRET change occurs when only 10% ATP is converted to ADP, demonstrating the excellent selectivity of the ADP antibody and the compatibility of the detection mixture in determining initial reaction rates of SERCA. Figure 4 (B) shows the calibration curves under different ATP and Ca^{2+} concentrations with the optimized detection mixture that contained 4 nM antibody and 63 nM tracer. When ATP concentration was above 4 μM or below 1 μM, the maximum assay window was not optimum. In the range of 4 μM to 1 μM, the curves had the steepest slope and the assay window was optimized around 0.1 units of FRET signal. The three curves obtained at 4 μM ATP and different concentrations of Ca^{2+} overlapped with each other. These results lead us to conclude that the FRET signal only depends on the ATP to ADP conversion, and has no

dependence on the Ca^{2+} concentration. This is important since Ca^{2+} concentration is a key parameter that is often varied when studying SERCA function.

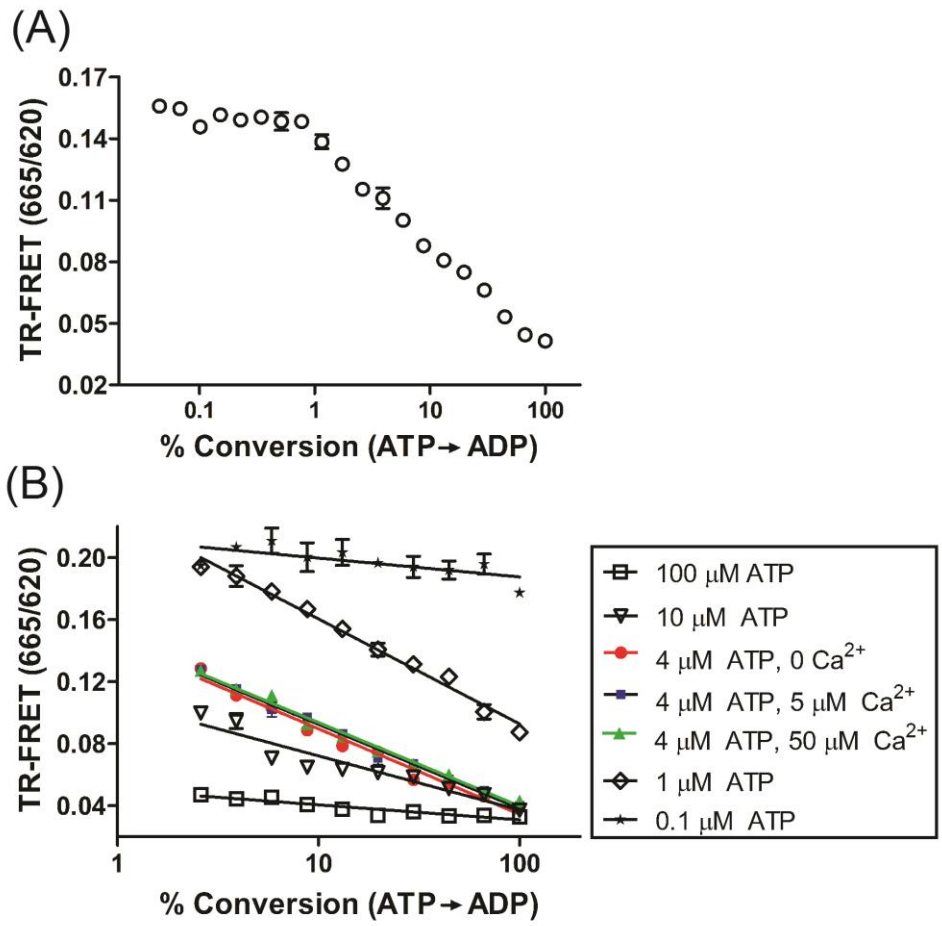

Figure 4. (A) FRET signals at different ATP to ADP ratios. The total concentration of adenosine in each sample was 2 μM. Detection mixture contained 4 nM ADP antibody and 63 nM ADP tracer. (B) Calibration curves showing FRET signals at different ATP to ADP ratios. Curves with black symbols and lines were obtained with different starting ATP concentrations in the absence of Ca^{2+}; curves with colored symbols and lines were obtained with varying concentrations of Ca^{2+} in the presence of 4 μM ATP. Each point is the average FRET value of three replicate samples. Error bars represent standard deviation.

SERCA Activity Assay

SERCA concentration was titrated to determine the lowest concentration that would provide a robust change in FRET signal in a reasonable reaction time. As shown in Figure 5 (A) and (C), SERCA samples with the concentrations spanning over 4 orders of magnitude were allowed to react with 4 μM of ATP at saturating Ca^{2+} concentrations in detergent and reconstituted lipids, respectively. Under ideal conditions, the FRET signal would

decrease at least ~80% of the maximum assay window within a reasonable reaction time. 5 nM SERCA was chosen to perform the activity assay in detergent since an ~80% decrease was observed for a 5 min reaction and a ~95% decrease was observed for a 18 min (Figure 5 (A)) reaction. The reaction proceeded more quickly in reconstituted lipids allowing the SERCA concentration to be reduced to 500 pM, with an ~80% decrease in signal observed after 5 min and a ~95% decrease after 20 min (Figure 5 (C)). Compared with the previously reported coupled enzyme assay, our method reduced the concentration of SERCA required to perform the assay by 5-fold in $C_{12}E_8$ and 50-fold in reconstituted lipids.[23, 24]

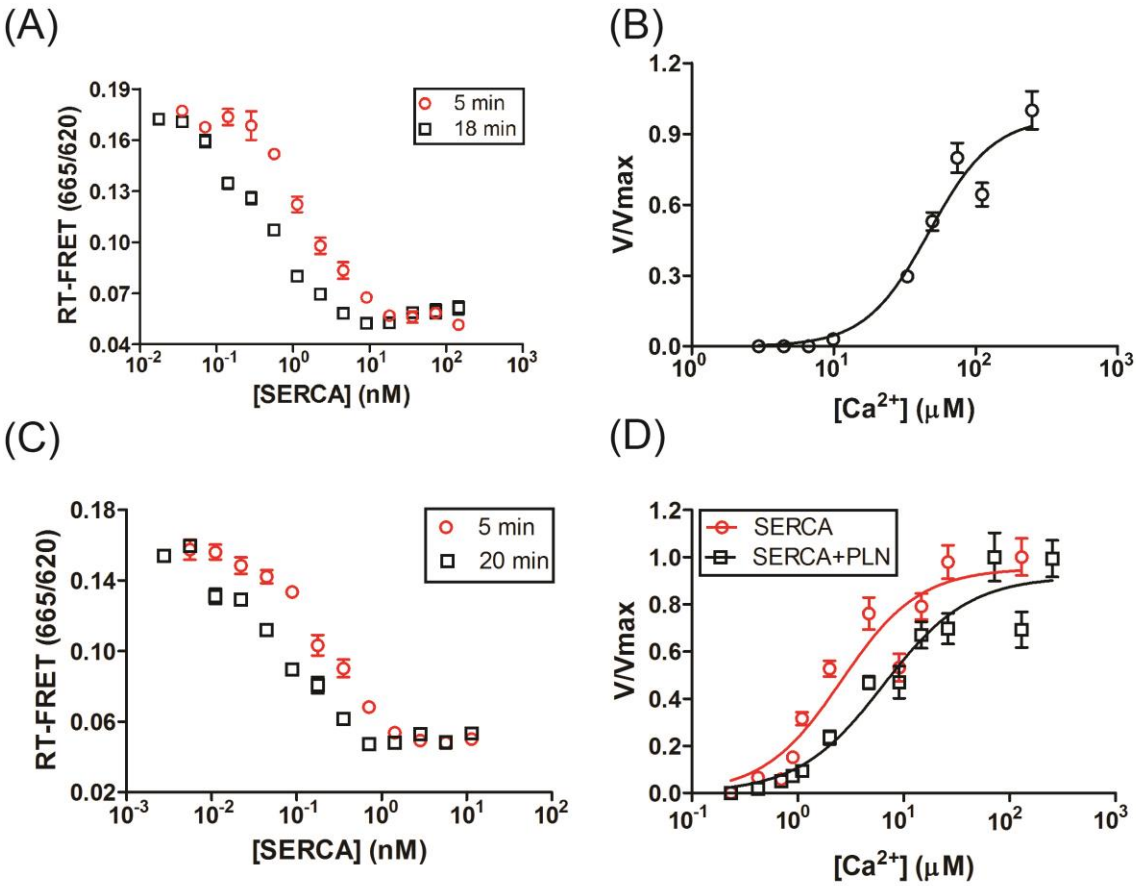

Figure 5. (A) SERCA titration curves in $C_{12}E_8$. Reactions were quenched at 5 min and 18 min. (B) Normalized SERCA activity curve in $C_{12}E_8$. (C) SERCA titration curves in reconstituted lipids. Reactions were quenched at 5 min and 20 min. (D) Normalized SERCA and SERCA+PLN activity curves in reconstituted lipids. Each data point shows the average FRET value of three parallel samples, and error bars represent standard deviation.

SERCA activity assays were finally performed in 4 μM ATP and 5 nM SERCA in $C_{12}E_8$ or 500 pM SERCA in reconstituted lipids. Reactions were quenched with the same volume of the optimized detection mixture to reach final concentrations of 2 μM adenosine, 4 nM antibody, and 63 nM tracer. Five reaction times were chosen with

three experiments performed for each reaction time, and initial reaction rates were determined from linear regression. Shown in Figure 5 (B) and (D) are SERCA activity curves obtained with varying [Ca^{2+}] concentrations. Although the maximum initial reaction rate (V_{max}) varied from day to day in both curves, these normalized curves demonstrate very reproducible K_{Ca} values. The curve in Figure 5 (B) has a K_{Ca} value of 47 ± 6 µM, compared to K_{Ca} values of 29 ± 13 µM and 32 ± 7 µM obtained from another two batches of SERCA sample (errors represent standard deviation). The SERCA curve shown in Figure 5 (D) has a K_{Ca} value of 3 ± 0.5 µM, compared to K_{Ca} values of 4.1 ± 0.5 µM and 4 ± 1 µM from another two additional reconstituted samples (errors represent standard deviation). When PLN was added, the midpoint of the curve shifted to higher Ca^{2+} concentrations, a result of the expected inhibition of PLN on SERCA activity. The shift of pK_{Ca} (ΔpK_{Ca}) in Figure 5 (D) is 0.40 ± 0.17, compared to ΔpK_{Ca} values of 0.48 ± 0.7 and 0.45 ± 0.13 from another two assays in reconstituted lipids performed on different days (errors represent standard deviation), demonstrating excellent inter day and inter sample reproducibility. Observed K_{Ca} and ΔpK_{Ca} values were comparable to those previously reported using PK assays.[25]

It is important to note the low concentrations of SERCA (500 pM) and PLN (25 nM) used to generate the inhibition curves shown in Figure 5 (D). Clear inhibition is demonstrated in Figure 5 (D), suggesting a significant fraction of SERCA is bound by PLN even at these low concentrations. The low nM to pM concentrations of SERCA and PLN used in this assay offer a promising approach for estimating the affinity of these two binding membrane proteins, which has proven to be challenging using existing assays.[34]

Conclusions

In conclusion, we have developed a sensitive assay for SERCA activity using the Transcreener® ADP^2 Assay TR-FRET Red kit. The optimized detection mixture contains 4 nM ADP antibody and 63 nM ADP tracer, allowing assays to be performed at 4 µM ATP with a maximum assay detection window. Our approach reduced the SERCA concentration required to perform the assay 5-fold in detergent preparations and 50-fold in reconstituted lipid preparations when compared to existing coupled enzyme assays. The sample volume was also decreased from 200 µL to 10 µL, yielding a 100-fold reduction in the mass of SERCA required to perform the assay in detergent preparations ($C_{12}E_8$) and a 1000-fold reduction when performed in reconstituted lipids. Moreover, the observed SERCA K_{Ca} values and pK_{Ca} shifts in the presence of PLN proved to be very reproducible across multiple preparations performed on multiple days. Finally, the greatly improved sensitivity offers additional information on

SERCA-PLN binding. The K_d of the SERCA-PLN complex was estimated to be approximately three orders of magnitude smaller than previous estimates.[34]

Acknowledgments

The authors gratefully acknowledge the National Institutes of Health (GM063533, GM64742 and GM72701) for financially supporting this work.

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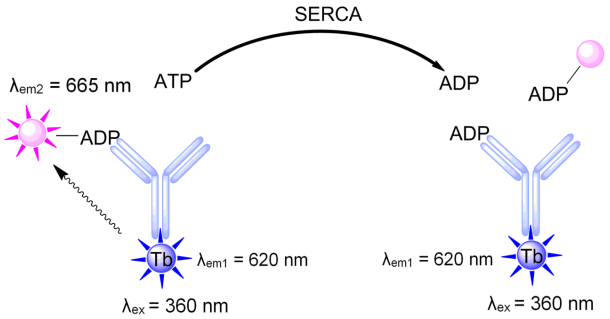
TOC Entry:



A SERCA activity assay which detects ADP production via time-resolved FRET is reported, improving sensitivity 50-fold and sample volume 1000-fold.