Chapter 18

Measurement of Plasma Membrane Calcium–Calmodulin-Dependent ATPase (PMCA) Activity

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Abstract

The plasma membrane calcium–calmodulin-dependent ATPase (PMCA) is a calcium-extruding enzymatic pump that ejects calcium from the cytoplasm to the extracellular compartment. Although in excitable cells such as skeletal and cardiac muscle cells PMCA has been shown to play only a minor role in regulating global intracellular calcium concentration, increasing evidence points to an important role for PMCA in signal transduction, in particular in the nitric oxide signaling pathway. Moreover, recent evidence has shown the functional importance of PMCA in mediating cardiac contractility and vascular tone. Here we describe a method in determining PMCA activity in the microsomal membrane preparation from cultured cells that overexpress specific isoform of PMCA by using modified coupled enzyme assay.

Key words: Calcium pump, microsome, ATPase, enzyme assay.

1. Introduction

The plasma membrane calcium–calmodulin-dependent ATPase (PMCA) is a ubiquitous calcium transporter that pumps calcium from the cytoplasm to the extracellular matrix. In most cell types, the role of PMCA is crucial in maintaining low intracellular calcium concentration. Four different PMCA genes (named PMCA1–4) and more than 20 splice variants have been described.

The structure of PMCA, isoform diversity, splice variants, and tissue distribution have previously been reviewed in great detail (1).

Unlike in non-excitable cells, where PMCA plays a major role in calcium extrusion, in excitable cells such as cardiac and skeletal muscle PMCA has only a minor role in the regulation of cytosolic calcium. In these cells, calcium is mainly removed through the sarcoplasmic reticulum calcium ATPase (SERCA) and the sodium calcium exchanger (NCX) (2). However, increasing evidence suggests that PMCA plays a more important role in signal transduction. Two main characteristics of a signaling molecule displayed by PMCA are the ability to interact with signaling proteins (3) and its localization in caveolae (4). Recent evidence from our laboratory suggested that PMCA has a role in mediating nNOS activity (5, 6) and demonstrated the physiological relevance of this novel signaling complex in regulating cardiac contractility (7, 8).

As PMCA plays an essential role in cell function development of an assay to measure its activity is very important. The use of human erythrocyte ghosts was common in the preparation of purified PMCA for this purpose (9). However, this will yield mainly PMCA4 as this is the main isoform expressed in human red blood cells. Moreover, a large amount of human blood is needed to obtain a substantial amount of good-quality purified PMCA. In this chapter we describe a method for measuring PMCA activity in the microsomal membrane preparation from cultured cells that overexpress PMCA. Measurement of PMCA activity is based on a coupled enzyme ATPase activity. The coupled enzyme assay is based on regeneration of PMCA-dependent release of ADP by pyruvate kinase, which converts phosphoenolpyruvate to pyruvate. Then, pyruvate is converted to lactate by lactate dehydrogenase using NADH (*see* **Fig. 18.1**). Detection of NADH decline
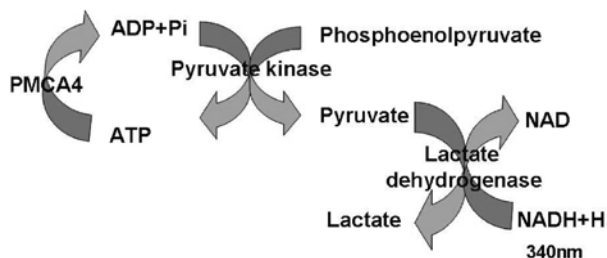

Fig. 18.1. Schematic diagram of PMCA activity assay. The phosphorylation of the aspartate residue in the catalytic domain of PMCA by ATP results in the generation of ADP + Pi. Then, the ADP is used by pyruvate kinase to generate pyruvate from phosphoenolpyruvate. Pyruvate produced by this reaction will be converted to lactate by lactate dehydrogenase. This reaction is coupled to the oxidation of NADH to NAD. The degradation of NADH can be monitored at 340 nm wavelength.

rate at 340 nm will determine the activity of the pump. An adaptation of this method in 96-well format is also discussed, which could be useful for the development of a high-throughput assay system.

2. Materials

2.1. Cell Culture and Infection with Adenovirus

1. Dulbecco's modified Eagle's medium (DMEM).
2. Fetal bovine serum (FBS).
3. Penicillin/streptomycin.
4. Non-essential amino acids.
5. Human embryonic kidney cells (HEK293) (ATCC).
6. Trypsin–EDTA (1X) liquid (0.05% trypsin, 0.53 mM EDTA tetra sodium salt).
7. AdEasy Adenoviral Vector System.
8. BJ5183 electroporation competent *Escherichia coli* cells.
9. LB broth medium.
10. Lipofectamine^{TM} 2000.
11. PMCA4 recombinant adenovirus (*see* **Section 3** for the cloning and generation of adenovirus) (*see* **Note 1**).

2.2. Microsomal Membrane Preparation

1. Harvest solution: 1X PBS, 0.26% 2 mg/ml aprotinin, 0.11% 2 mg/ml leupeptin, and 0.1% 0.1 M phenylmethylsulfonyl fluoride (PMSF).
2. Hypotonic solution: 10 mM Tris–HCl, pH7.5, 1 mM $MgCl_2$, 0.5 mM EGTA, 2 mM DTT, 0.2% 2 mg/ml aprotinin, and 0.05% 2 mg/ml leupeptin.
3. Homogenate solution: 10 mM Tris–HCl, pH 7.5, 2 mM DTT, 0.38 M sucrose, 0.3 M KCl, 0.2% 2 mg/ml aprotinin, and 0.05% 2 mg/ml leupeptin.
4. Final solution: 10 mM Tris–HCl, pH 7.5, 1 mM DTT, 0.19 M sucrose, 0.15 M KCl, 0.2% 2 mg/ml aprotinin, 0.05% 2 mg/ml leupeptin, and 0.02 mM $CaCl_2$.

2.3. Coupled Enzyme Assay

1. Coupled enzyme assay reaction mixture: 50 mM HEPES–Tris, pH 7.4, 160 mM KCl, 2 mM $MgCl_2$, 5 mM NaN_3, 1 μg/ml alamethicin, 1 mM ATP–Tris, 1 mM phosphoenolpyruvate, 1 U/ml pyruvate kinase, 0.6 mmol/L NADH, 1 U/ml lactate dehydrogenase.

2. Calcium and calmodulin mixture: 5 µg of calmodulin and 4 µM-free calcium (*see* **Note 2**).

3. Spectrophotometer able to read at 340 nm wavelength.

4. Microplate reader with 340 nm wavelength filter.

3. Methods

3.1. Cell Culture for HEK293 Cells

Maintained human embryonic kidney (HEK293) cells in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, and 1% non-essential amino acids. Cells are passed when they reach 75–90% confluence. To passage the cells, remove the medium from the tissue culture flask and wash the cells twice with phosphate-buffered saline (PBS). Then, add 5 ml of trypsin–EDTA (1X) liquid to each flask and incubate for 5 min at 37°C. To neutralize trypsin, add 10 ml of the culture medium to each flask. Cells from one flask are split either into two or three flasks and topped up to 25 ml medium in each flask.

3.2. Generation of Recombinant Adenovirus for PMCA Overexpression

1. AdEasy Adenoviral Vector System is used to clone the PMCA4b cDNA to the adenoviral vector. The PMCA4b cDNA is cloned to the shuttle vector pShuttle-CMV to obtain pShuttle-CMV-PMCA4b. As a control, an adenovirus overexpressing LacZ is used. To generate recombinant adenoviral vectors carrying CMV-PMCA4b or CMV-LacZ constructs, linearize pShuttle-hPMCA4b and pShuttle-CMV-LacZ using *Pme*I restriction enzyme. Precipitate linearized vectors (using three volumes of absolute ethanol), then dephosphorylate, and gel purify.

2. Mix 1 µg of linearized/dephosphorylated shuttle vector and 100 ng of pAdEasy-1 with 40 µl BJ5183 electro-competent *E. coli* on ice.

3. Electroporate cells with a single pulse using Gene-pulser set at 200 Ω, 2.5 kV, and 25 µF.

4. Add 1 ml of LB broth medium to each transformation and incubate at 37°C for 1 h.

5. Plate cells onto LB agar plates containing 50 µg/ml kanamycin and incubate overnight at 37°C.

6. Select small colonies from the plates (large colonies usually represent re-linearization of the shuttle vector). Grow selected colonies in small scale cultures (~5 ml of LB broth with kanamycin).

7. Check plasmids for homologous recombination by digestion with *Pac*I on a 0.75% agarose gel.

3.3. Generation of Recombinant Adenovirus Particles

1. Plate HEK293 cells at a density of 0.7×10^6 cells per 25 cm^2 flask 24 h prior to transfection.

2. Transfect 5 µg of linearized adenoviral plasmids into HEK293 cells using 10 µl Lipofectamine$^{\text{TM}}$ 2000.

3. Replace the medium 24 h after transfection and then every 48 h. Cells producing adenovirus will appear as patches of rounding dying cells. These cells will lyse and release adenoviral particles which subsequently infect neighboring cells, then plaques start to form. When the majority of cells are detached, a primary stock of adenovirus can be prepared.

4. Collect floating and attached cells in the growth medium by pipetting it up and down.

5. Pellet cells by centrifugation ($1,000 \times g$ for 5 min).

6. Resuspend each flask of cells in 0.5 ml PBS and then perform four rounds of rapid freezing and thawing by transferring between a dry ice/methanol bath and a 37°C water bath (for 5 min each).

7. Remove cell debris by centrifugation at $13,000 \times g$ for 10 min. The supernatant contains the recombinant adenovirus.

8. Use 10 µl of the primary stock to infect a 175 cm^2 flask of 70% confluent HEK293 cells, change culture medium every 2–3 days until plaques formed.

9. Once the majority of cells have detached, harvest the cells and isolate adenovirus by freeze–thawing. Use the secondary stock to infect 20 flasks of HEK293 cells and repeat the procedure above to provide a tertiary stock.

10. Use freezing and thawing method to purify the adenovirus particles from the tertiary stock (*see* **Note 3**).

11. In order to determine the number of adenovirus particles in the tertiary preparations, plate HEK293 cells at 5×10^3 cells/well (in 96-well plate) in a total volume of 100 µl medium. After 24 h remove the medium and replace with 100 µl of serially diluted adenovirus stocks (1×10^{-2} to 7.63×10^{-12}).

12. Analyze each dilution in triplicate. After 24 h, add 100 µl of fresh DMEM (with 10% FBS) to each well. Change the medium on day 4 and monitor plaque formation each day.

13. Eight days after infection, use the final dilution which shows plaque formation as the endpoint of the assay. From knowing the number of adenovirus particles the infection process can be standardized (*see* **Table 18.1**).

Table 18.1
Determination of adenovirus PFU from serial dilutions

Dilution	PFU/mL	Dilution	PFU/mL
10^{-2}	1×10^3	7.81×10^{-9}	1.28×10^9
10^{-3}	1×10^4	3.91×10^{-9}	2.56×10^9
10^{-4}	1×10^5	1.95×10^{-9}	5.12×10^9
10^{-5}	1×10^6	9.77×10^{-10}	1.02×10^{10}
10^{-6}	1×10^7	4.88×10^{-10}	2.05×10^{10}
5×10^{-7}	2×10^7	2.44×10^{-10}	4.1×10^{10}
2.5×10^{-7}	4×10^7	1.22×10^{-10}	8.19×10^{10}
1.25×10^{-7}	8×10^7	6.1×10^{-11}	1.64×10^{11}
6.25×10^{-8}	1.6×10^8	3.05×10^{-11}	3.28×10^{11}
3.12×10^{-8}	3.2×10^8	1.53×10^{-11}	6.55×10^{11}
1.56×10^{-8}	6.4×10^8	7.63×10^{-12}	1.31×10^{12}

3.4. Microsomal
Membrane
Preparation

1. Plate HEK293 cells in 100 mm tissue culture plates, at a density of 4.5×10^6 cells/plate, 24 h prior to virus infection.

2. Infect cells in 10 ml of DMEM plus 10% FBS by 25 MOI (*see* **Note 1**) of the indicated recombinant adenovirus, PMCA4b or LacZ (control) virus, for 48 h.

3. After 48 h, remove the medium and wash cells three times with PBS.

4. After washing, harvest cells in 5 ml of harvest medium and then centrifuge cells at $3000 \times g$ for 10 min at 4°C.

5. Incubate the cell pellet in 3 ml hypotonic solution for 10 min in ice.

6. Then homogenize cells using Dounce homogenizer for 40 strokes before the addition of 3 ml homogenate solution.

7. Homogenize cells slowly for another 20 strokes to seal the vesicles.

8. Spin cell homogenate at $3500 \times g$ for 20 min to remove cell debris.

9. Then, add 60 μl 0.25 mM EDTA and 1.08 ml of 2.5 M KCl to the supernatant.

10. Centrifuge supernatant at $100{,}000 \times g$ for 40 min at 4°C to pellet the microsomes. Finally resuspend the pellet in 0.4 ml final solution and measure protein concentration using standard bicinchoninic acid (BCA) protein assay kit (*see* **Note 4**).

3.5. Coupled Enzyme Assay Measuring Ca^{+2}-Dependent ATPase Activity

1. All measurements are carried out at 37°C using Ultrospec 3000 Spectrophotometer setup at 340 nm wavelength.

2. Add 500 µl of coupled enzyme assay reaction mixture to a quartz cuvette and pre-warmed to 37°C. Then, record the baseline activity for 20 min after addition of 20 µg microsomal preparation to the reaction mixture.

3. To start the ATPase activation, add 4 µM calcium followed by the addition of 5 µg calmodulin to the reaction. Then, record the decrease of NADH at 340 nm absorbance over 5 min following each addition.

4. To stop the Ca^{+2}-dependent ATPase activity, add 2 mM EGTA and follow the reaction for another 5 min.

5. To stop the calcium- and magnesium-dependent activity, add 1 mM EDTA to the reaction mixture.

6. The Ca^{+2}/calmodulin-dependent ATPase activity can be calculated by subtracting the fitted slopes. **Figure 18.2** described representative trace with control microsomes and **Fig. 18.3** described representative trace with microsomes from cells overexpressing PMCA4.
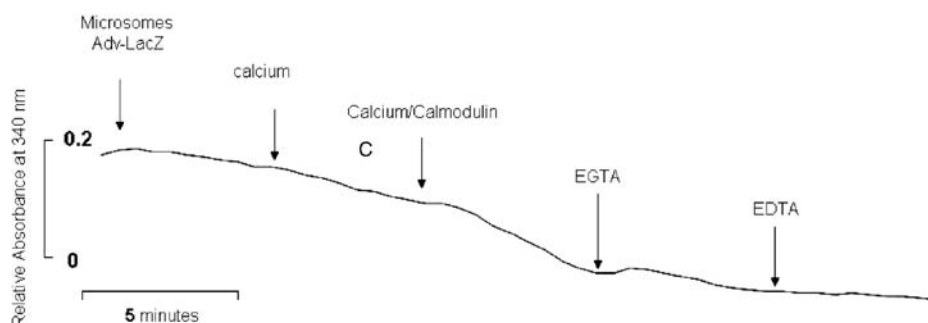

Fig. 18.2. Representative trace of ATPase assay using microsomal particles from cells infected with LacZ overexpressing virus as a negative control. The decrease of absorbance at 340 nm was mainly due to spontaneous degradation of NADH. No significant changes were observed after the addition of either calcium or calcium–calmodulin.

3.6. Adaptation of the Assay in 96-Well Format

We have optimized this assay to be conducted in a 96-well format (*see* **Fig. 18.4**). A microplate reader with 340 nm filter is needed. For controls, prepare wells containing microsomes only, microsomes + calcium, and microsomes + calcium and calmodulin.

Protocol:

1. All the measurements are carried out at 37°C in an Ascent microplate reader.

2. Add 250 µl of coupled enzyme assay reaction mixture in each well (pre-warm to 37°C). Record the baseline activity for 30 min after addition of 10 µg microsomal preparation
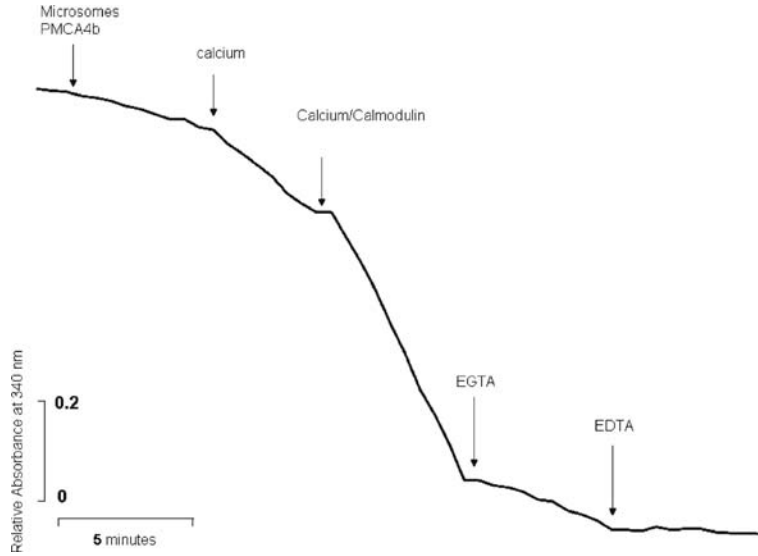
Fig. 18.3. Representative trace of ATPase assay using microsomal particles from cells infected with PMCA4 overexpressing virus. The decrease of absorbance at 340 nm after addition of calcium–calmodulin represented the calcium–calmodulin-dependent ATPase, which was mainly due to the PMCA4 activity.
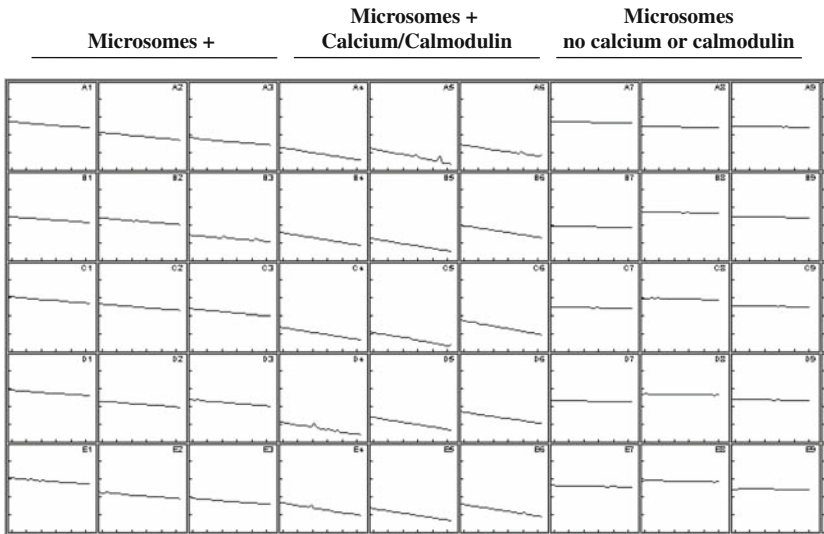

Fig. 18.4. Representative trace from 96-well format PMCA assay. About 20 μg of microsomes from cells overexpressing PMCA4b was added to each well. In columns 1–3, PMCA4b was activated with 4 μM free calcium. In columns 4–6, the pump was activated with 4 μM free calcium, and 5 μg calmodulin. Columns 7–9 served as negative controls.

to 250 μl of the reaction mixture. Monitor the reduction of NADH by automatic scanning setup at 30 s intervals.

3. To start the ATPase activation, add calcium and calmodulin mixture to the reaction and then follow the 340 nm absorbance for another 30 min.

4. Add substance (inhibitor/activator) to be tested and record the absorbance at 340 nm for another 30 min.

5. To stop the Ca^{+2}-dependent ATPase activity, add 2 mM EGTA and continue the reaction for another 15 min.

6. Calculate the Ca^{+2}/calmodulin-dependent ATPase activity using the equation below. Use the fitted slopes in each step to determine the effects of each substance added.

3.7. Data Analysis

Enzyme activity is expressed as the rate of NADH reduction per milligram microsomal protein. It can be calculated using the following equation:

$$E = \left(\frac{-S}{6.22 \times P} \right) \times 250$$

where E = enzyme activity (mM NADH/min/mg protein)

S = fitted slope (per minute) at the last 10 min of each measurement

6.22 is millimolar extinction coefficient of NADH at 340 nm and 37°C

P = microsomal protein added (in milligram protein)

250 is the reaction volume in μl

4. Notes

1. The amount of adenovirus added in the experiments depends on the number of cells plated. For example, addition of similar number of adenovirus particles to the cells will give a multiplicity of infection (MOI) of 1. In most experiments, we infected cells with an MOI of 25, meaning the number of adenovirus particles added was 25 times more than the number of cells plated. Cells were infected for 48 h before harvesting.

2. Free calcium concentration in the solution is calculated using Fabiato equation. A free software to calculate the free calcium (MaxChelator) can be downloaded from the following website: http://www.stanford.edu/~cpatton/downloads.htm.

3. Purified adenovirus tertiary stock needs to be aliquoted in small volume (30 μl) and stored at –80°C. We have found

that repeated freeze and thaw of the virus stock will reduce the virus viability.

4. The microsomes are resuspended in the final solution at the concentration of 2 mg/ml and should be stored as 200 µl aliquots in liquid nitrogen. Storing the microsomes in liquid nitrogen will preserve the activity better than storing in 80°C freezer.

Acknowledgments

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