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Original article

Development and characterization of a novel fluorescent indicator protein PMCA4-GCaMP2 in cardiomyocytes

Tamer M. A. Mohamed [a,b,1], Riham Abou-Leisa [a,1], Florence Baudoin [a], Nicholas Stafford [a], Ludwig Neyses [a,2], Elizabeth J. Cartwright [a,2], Delvac Oceandy [a,*,2]

[a] Institute of Cardiovascular Sciences, University of Manchester, Manchester Academic Health Sciences Centre, Manchester M13 9PT, UK
[b] Department of Biochemistry, Faculty of Pharmacy, Zagazig University, Zagazig, Egypt

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ABSTRACT

Isoform 4 of the plasma membrane calcium/calmodulin dependent ATPase (PMCA4) has recently emerged as an important regulator of several key pathophysiological processes in the heart, such as contractility and hypertrophy. However, direct monitoring of PMCA4 activity and assessment of calcium dynamics in its vicinity in cardiomyocytes are difficult due to the lack of molecular tools. In this study, we developed novel calcium fluorescent indicators by fusing the GCaMP2 calcium sensor to the N-terminus of PMCA4 to generate the PMCA4–GCaMP2 fusion molecule. We also identified a novel specific inhibitor of PMCA4, which might be useful for studying the role of this molecule in cardiomyocytes and other cell types.

Using an adenoviral system we successfully expressed PMCA4–GCaMP2 in both neonatal and adult rat cardiomyocytes. This fusion molecule was correctly targeted to the plasma membrane and co-localised with caveolin-3. It could monitor signal oscillations in electrically stimulated cardiomyocytes. The PMCA4–GCaMP2 generated a higher signal amplitude and faster signal decay rate compared to a mutant inactive PMCA4^{mut}GCaMP2 fusion protein, in electrically stimulated neonatal and adult rat cardiomyocytes. A small molecule library screen enabled us to identify a novel selective inhibitor for PMCA4, which we found to reduce signal amplitude of PMCA4–GCaMP2 and prolong the time of signal decay (Tau) to a level comparable with the signal generated by PMCA4^{mut}GCaMP2. In addition, PMCA4–GCaMP2 but not the mutant form produced an enhanced signal in response to β-adrenergic stimulation. Together, the PMCA4–GCaMP2 and PMCA4^{mut}GCaMP2 demonstrate calcium dynamics in the vicinity of the pump under active or inactive conditions, respectively.

In summary, the PMCA4–GCaMP2 together with the novel specific inhibitor provides new means with which to monitor calcium dynamics in the vicinity of a calcium transporter in cardiomyocytes and may become a useful tool to further study the biological functions of PMCA4. In addition, similar approaches could be useful for studying the activity of other calcium transporters during excitation–contraction coupling in the heart.

1. Introduction

The plasma membrane calcium/calmodulin dependent ATPase (PMCA) is a ubiquitous calcium transporting enzyme which mainly functions to extrude intracellular calcium (reviewed in [1]). In non-excitable cells PMCA is understood to play a major role in calcium extrusion [2], however in excitable cells (such as cardiomyocytes), its importance in respect to bulk calcium transport has traditionally been assumed to be minor. Using serial pharmacological inhibitors, several studies have shown that the majority of cytosolic calcium removal is via the sarcoplasmic reticulum ATPase (SERCA) and sodium/calcium exchanger (NCX) (reviewed in [3]). PMCA1 and PMCA4 are the two isoforms expressed in the cardiomyocytes [4] and their high affinity for calcium has led to speculation that they may participate in, or fine tune, diastolic calcium extrusion [5,6]. Based on an alternative hypothesis, our group and others have identified a signalling role for isoform 4 of the PMCA (PMCA4). PMCA4 interacts with and modulates the activity of other molecules, which have significance in cardiac physiology. For example, PMCA4 modulates cardiac contractility and hypertrophy by regulation of neuronal nitric oxide synthase (nNOS) and calcineurin, respectively [7–10]. Both nNOS and calcineurin are calcium-dependent enzymes and it is therefore logical to speculate that PMCA4 may be important in defining calcium concentration within its vicinity and hence alter the activity of its interacting partners.

Abbreviations: PMCA, plasma membrane calcium/calmodulin dependent ATPase; NCX, sodium/calcium exchanger; SERCA, sarcoplasmic reticulum ATPase; nNOS, neuronal nitric oxide synthase; GECI, genetically encoded calcium indicator; GCaMP2, GFP-calmodulin-based GECI; NRCM, neonatal rat cardiomyocytes; ATA, aurintricarboxylic acid.

* Corresponding author at: Institute of Cardiovascular Sciences, University of Manchester, Manchester Academic Health Sciences Centre. Room 1.302 Stopford Bldg, Oxford Road, Manchester M13 9PT, UK. Tel.: +44 161 2751772; fax: +44 161 2755669.
E-mail address: Delvac.oceandy@manchester.ac.uk (D. Oceandy).

[1] Equally contributed to the work.
[2] L.N., E.J.C. and D.O. are equal senior authors.

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Considering that PMCA4 might exert its signalling role by regulating local calcium concentration [7,10,11], it is important to develop a novel tool to assess calcium dynamics in the vicinity of PMCA4. In this study we fused PMCA4 with a genetically encoded calcium indicator (GECI) [12,13] and characterized its properties in situ in cardiomyocytes. We used the GFP-based GECI (GCaMP2), which exploits the calcium/calmodulin dependent rearrangement of recombinant GFP that results in an increase in fluorescence intensity upon binding with calcium/calmodulin [14]. It has been shown in a number of models that GCaMP2 fluorescent intensity is a function of calcium concentration [14]. It was also reported previously that using transgenic mouse technology, expression of GCaMP2 in the heart can be used to examine calcium transients in vivo [13]. The high brightness and stability of GCaMP2 enable the measurement of the rapid changes in Ca^{2+} transients in all regions of the beating mouse heart and for prolonged pacing and mapping studies in isolated, perfused hearts [13]. Here we used an adenoviral system to enable the delivery of GCaMP2 in isolated/cultured cardiomyocytes and assess the ability of this adenoviral-based GCaMP2 to detect rapid calcium fluctuations in isolated cardiomyocytes. We also generated adenoviruses to enable expression of two fusion molecules: PMCA4–GCaMP2 and PMCA4^{mut}GCaMP2. In addition, through screening a medically optimised chemical library we have identified a new and highly selective PMCA4 inhibitor, which was very useful in studying the fusion fluorescent molecules. Our analyses suggest that the indicators were able to report calcium dynamics in the local vicinity of PMCA4 which was dependent on PMCA4 activity.

2. Methods

2.1. Adenovirus

Adenoviruses expressing the cytoplasmic GCaMP2 (cytoplasmic calcium sensor) [13] were generated by cloning GCaMP2 (a generous gift from J Nakai, Saitama, Japan) to the pAd/CMV/V5-DEST vector (Invitrogen) using the Gateway system (Invitrogen) following the manufacturer's instructions. To generate adenovirus expressing GCaMP2 linked to PMCA4 or PMCA4^{mut}, we cloned GCaMP2 cDNA to the N terminal end of PMCA4 or PMCA4^{mut} and then subcloned the resulting construct to the pAd/CMV/V5-DEST vector as above.

2.2. Isolation of adult and neonatal cardiomyocytes and western blot analysis

Adult cardiomyocytes were isolated from 2 to 3-month old rats. In brief, rats were sacrificed by cervical dislocation, the hearts were rapidly removed and then perfused via the aorta with isolation solution pH 7.34 (134 mM NaCl; 11 mM Glucose; 4 mM KCl; 1.2 mM MgSO4; 1.2 mM NaH2PO4; 10 mM HEPES) for 4 min followed by 9 min perfusion with a solution containing 0.6 mg/ml and 0.075 mg/ml of collagenase type II (Worthington) and proteases type XIV (Sigma-Aldrich), respectively. Hearts were then perfused with Tyrode solution containing 50 mM taurine pH 7.34 for 12 min. The ventricles were cut from the heart and placed in Tyrode–taurine solution. The ventricles were then cut in half and pipetted up and down through a Pasteur pipette in 5 ml of Tyrode–taurine solution to release the cardiomyocytes. The cardiomyocytes were then plated on laminin coated coverslips, infected with adenovirus and cultured for 48 h using a previously described protocol [15].

Neonatal rat cardiomyocytes (NRCM) were isolated from 1 to 3-day old Sprague Dawley rat neonates using a protocol as described previously [16]. Western blot analyses using anti-GFP (Abcam) and anti-GAPDH (Abcam) (loading control) were carried out as described before [17].

2.3. Immunofluorescence

Neonatal or adult rat cardiomyocytes were plated on laminin coated coverslips. Cells were washed twice with PBS and then fixed by adding 0.5 ml 4% formaldehyde for 15 min. Fixed cells were washed three times with PBS then permeabilised with 0.1% Triton X-100 for 15 min and blocked with 10% horse serum in PBS for 30 min at room temperature. Cells were then probed with monoclonal mouse FITC conjugated anti-GFP (Abcam) or monoclonal mouse anti-caveolin-3 antibodies (BD) (1/100) diluted in 1% horse serum for 1.5 h at room temperature, washed three times in PBS and then labelled with TR conjugated anti-mouse (1/200) (Jackson Lab) diluted in 1% horse serum for 1.5 h in the dark. The cells were then washed a further three times in PBS and once in water. Coverslips were mounted onto slides using a Prolong Antifade™ Kit (Molecular Probes) and cell staining was visualized using a Leica SP5 confocal microscope.

2.4. Calcium transient recordings using GCaMP2, PMCA4-GCaMP2 and PMCA4^{mut}GCaMP2

NRCM or adult rat cardiomyocytes were infected with adenovirus for the specific calcium sensor for 48 h. In order to measure calcium transients the coverslips containing infected myocytes were placed in a bath with a cover slip base. This bath was positioned on the stage of an epifluorescence adapted inverted Olympus IX70 microscope fitted with an Olympus America camera. The myocytes were perfused with Tyrode solution and then field stimulated by two silver wire electrodes initiating an electrical current at a frequency of 1 Hz. Calcium oscillations during myocyte contraction were recorded at 33 °C. Excitation was set at 485 nm using an ARC lamp (Cairn Inc), by means of a laser spot applied to small areas of the cell. The fluorescence emission at 525 nm was collected using a dichroic filter and a Cairn Integra photomultiplier tube. The data were collected and analyzed using Ionwizard software (Ionoptix Inc.). The calcium transients signal was represented as a ratio of the post-stimulation fluorescence, F, divided by the mean pre-stimulation baseline fluorescence, F_0 for each cell. The single exponential tau was calculated using the Ionwizard software (Ionoptix Inc.) as the exponential decay time constant of the calcium transients following the exponential fitting of the calcium transients decay slope and calculated as tau $= 1/K_{fall}$ where K_{fall} = exponential rate constant of the calcium decay. Caffeine experiments were carried out by perfusing the cardiomyocytes with normal Tyrode solution (TS). Then cells were rapidly perfused with 10 mM caffeine for 3 min using an 8 channel local rapid perfusion system (MPER8 (Cairn research LTD)) which was placed in close proximity to the cell to ensure rapid application of caffeine. For 0Na/0Ca experiments the cells were perfused with Na^+ and Ca^{2+}-free Tyrode solution (0Na0Ca), in which all sodium salts were replaced with lithium salts, for 60 s prior to caffeine application. Indo-1 experiments were performed following methods described elsewhere [7,17].

2.5. Microsomal preparation and coupled enzyme assay for PMCA4 activity

HEK293 cells were infected with the PMCA4 [7], PMCA4–GCaMP2, PMCA4^{mut}GCaMP2, GCaMP2, or LacZ adenovirus for 48 h. Microsomal membrane preparations were carried out from the transfected cells as follows: cells were washed three times with PBS and then harvested in 5 ml harvest solution (1× PBS, 0.26% 2 mg/ml aprotinin, 0.11% 2 mg/ml leupeptin, 0.1% 0.1 M PMSF). Harvested cells were centrifuged at 1000 ×g for 10 min at 4 °C. 3 ml hypotonic solution (10 mM Tris–HCl, pH 7.5, 1 mM MgCl2, 0.5 mM EGTA, 2 mM DTT, 0.2% 2 mg/ml aprotinin, and 0.05% 2 mg/ml leupeptin) was added to the cell pellet for 10 min on ice. Swelled cells were homogenised in a Dounce homogenizer before addition of 3 ml 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); homogenization was completed to seal the