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Measurement of phosphorylated phospholamban levels in cardiomyocytes (HL-1) by immunoprecipitation

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Abstract

Phospholamban (PLN) is a key regulatory protein involved in cardiac calcium signaling through the pumping of cytoplasmic Ca^{2+} into the sarcoplasmic reticulum (SR). Recent systems-level studies have focused on integrating quantitative data (e.g. protein expression levels) for a better understanding of cardiac systems biology. In this view, we developed a capillary electrophoresis (CE) based immunoprecipitation method for the measurement of phospho-PLN (ser 16) in cardiomyocytes (HL-1 cell line). Dose-dependent isoproterenol (Iso) treated cells were analyzed using CE, and the phospho-PLN levels were quantified using specific polyclonal antibodies. The CE method employed was accurate, quick and easier compare to other techniques and the results are useful for the subsequent computational systems biology research.

Keywords: Capillary electrophoresis; Immunoprecipitation; Phospholamban; HL-1

1. Introduction

Cardiac hypertrophy describes an abnormal enlargement of the heart where several signaling pathways are routed [1]. It is also involved in various diseases such as myocardial infarction and arrhythmia. Intracellular hypertrophic signals are generated through macromolecular signaling complexes that are assembled around the cardiac ryanodine receptor (RyR2) [2]. Phospholamban (PLN) is a key regulatory protein of the sarcoplasmic reticulum (SR) Ca^{2+}-ATPase (SERCA2a) in cardiac SR, and is activated in response to different hypertrophic stimuli via cAMP-dependent kinase (protein kinase A) and calcium/calmodulin-dependent kinase (CaMKIII) pathways [3]. Studies have suggested that PLN is a significant determinant in the inotropic responses of the heart to beta-adrenergic stimulation. Pentameric phosphorylated PLN is inactive and the monomeric dephosphorylated form inhibits SR Ca^{2+} uptake by SERCA2a [4]. Interaction between PLN and SERCA in cardiac hypertrophy has generated a great deal of interest. Stoichiometry of PLB to SERCA is required for the regulation of calcium transport across SR and its role is unclear [5]. Although many studies focus on PLN and SERCA2a interactions, precise and accurate quantification data for these proteins are essential to understand their functional mechanisms. From a cardiac systems biology perspective, this data is a prerequisite for computational modeling which can help clarify the role of proteins in heart failure as a whole system.

To date, the routine method for measurement of protein levels is immunoblotting. Although definitive, this method cannot be employed for low molecular weight biomolecules including PLN, a 52 amino acid protein with a mass of 1666. Drago and Colyer have demonstrated the use of site-specific antibodies to determine an absolute specificity of phosphorylation and discriminate between phosphorylation sites of PLN [6]. Utilization of these antibodies allows quantification of the different phosphorylation states (ser 16 and thr 17) of PLN. Mayer et al. have reported on an immunoassay to determine the PLN levels in crude cardiac homogenates from the wild type and transgenic mice using mAb 1D11 antibodies [7]. Dot blots and Western blots employed in this study suffered from many disadvantages. For example, non-

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specific binding was detected, the process was time consuming and laborious, and quantification was relative concentrations. In order to accurately measure the PLN levels, a new method should be developed which can give absolute concentration and manifold vital information from a single experiment.

State-of-the-art research in analytical biochemistry has been greatly facilitated by recent advances in capillary electrophoresis (CE). Despite its numerous advantages, CE has seldom been used to determine the variety of biomolecules in tissues, cells or biological fluids [8]. Numerous reports have demonstrated the use of CE separation technique for immune based reaction studies. The main advantage of this technique is its high specificity in detecting biomolecules, as well as the fact that it is a rapid and sensitive technique. By detecting the antigen at its specific mass to charge ratio, artifacts due to antibody cross-reactivity can be overcome.

In this study, phosphorylated levels of PLN (ser 16), were measured using a synthetic phospho-peptide (PLN 13–26; $RRAS_pTIEMPQQARQ$) corresponding to the cytosolic part of the PLN monomer with the phosphorylation sites as the native PLN which phosphorylated by protein kinase A and CaMKII respectively. An immunoprecipitation (IP) procedure was used that is suitable for CE analysis of PLN. PLN was precipitated from HL-I cells extracts (or standard) by immunoprecipitation with antibodies directed against phospho-PLN (ser-16), and was then analyzed by CE.

2. Materials and methods

2.1. Chemicals

Synthetic phosphorylated PLN peptide ($RRAS_pTIEMPQQARQ$) was obtained from Any Gen Co. Ltd. (Gwang-ju, Korea). The purity of the phosphorylated peptide was confirmed by HPLC and mass spectrometry analysis. PLN (Phospho ser 16) rabbit polyclonal antibody was purchased from abcam (Cambridge, USA). Sepharose protein G beads were procured from Amersham Pharmacia Biotech. (Uppsala, Sweden). Isoproterenol (Iso) was from Sigma chemical (St. Louis, USA). All chemical reagents as well as solvents used for sample preparation, buffer preparation and analyses were analytical reagent grade. The deionized water used in samples preparation was obtained from a Milli-Q water purification system (Millipore Corp., Bedford, USA).

2.2. Buffer solutions

Buffer H was made from: 50 mM β-glycerolphosphate, 1.5 mM 1,2-bis-(2-dicarboxymethylaminoethoxy)ethane (EGTA), 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM dithiothreitol, 0.1 mM sodium orthovanadate, 1 mM benzamidine, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 2 μg/ml pepstatin A with a pH of 7.3. The cell lysis buffer A consisted of 50 mM Tris (pH 8.8), 50 mM NaCl, 0.5% NP-40, 2 mM EDTA and protease inhibitor cocktail. Tris-buffered saline tween-20 (TBST) consisted of 10mM Tris with a pH of 7.6, 137mM NaCl, and 0:1% Tween 20. The phosphate buffered saline (PBS) consisted of 137 mM NaCl, 27 mM KCl, 8.1 mM Na_2HPO_4, and 1.5 mM KH_2PO_4. The CE electrolyte was prepared by dissolving the appropriate amount of sodium phosphate (100 mM) in distilled deionized water while adjusting the pH (2.5–3.0) with dilute phosphoric acid.

2.3. Standardization of immunoprecipitation

Reaction I: A 200 μl portion of synthetic phospho-peptide or HL-1 cell lysate sample was incubated with anti-phospho-PLN (ser16) antibody in PBS at 4 °C overnight. A suspension of 20 μl sepharose protein G beads (washed with buffer H) was added to the mixture and further incubated for 2 h. Then the sample was centrifuged for 10 min at 15 000 ×*g*. All liquids were removed using a flat pipette tip and replaced by PBS. This washing procedure was done in triplicate. After the third wash, 20 μl of 0.5% TFA (v/v) was added to the beads, centrifuged for 15 min at 15 000 ×*g* and the supernatant was stored in a fresh vial. This elution step was repeated once again using 20 μl of 0.1% TFA (v/v) and the supernatants were pooled and stored at −20 °C until use.

Reaction II: A 20 μl portion of sepharose protein G beads (Amersham Pharmacia Biotech., Uppsala, Sweden) (washed with buffer H) was incubated with anti-phospho-PLN (ser16) antibody in PBS at 4 °C overnight. A 200 μl synthetic phospho-peptide or HL-1 cell lysate sample was added and the sample was incubated for 2 h. Then the sample was subjected to the same washing and elution procedure as in Reaction I.

2.4. HL-1 cell lysate preparation

The HL-1 cardiomyocytes cell line was provided by Prof. Do Han Kim at the Gwangju Institute of Science and Technology, Korea. The cells were maintained in Claycomb Medium (JRH Biosciences, USA) supplemented with 10% fetal bovine serum (JRH Biosciences, USA), 100 units/ml penicillin (Sigma, USA), 100 μg/ml streptomycin (antibiotic/antimycotic solution, Invitrogen, USA), 0.1 mM norepinephrine (Sigma, USA) and 2.0 mM L-glutamine (Sigma, USA). The supplemented medium was changed every 24 h. Standard incubation conditions of 37 °C, 5% CO_2, 95% air and 95% relative humidity were used. The culture cavity was pretreated with gelatin and fibronectin (3 ml/flask) for 1 h after which the cells (passages F5P61) were plated in a petri dish at a density of 1500 K cells/dish and cultured until they reached confluence in the Claycomb medium. Following treatments with different concentrations of Iso for 30 min, the cells were scraped off the plate and pelleted by centrifugation at 1200 ×*g* for 30 min at 4 °C. The cells were washed with PBS and then lysed by incubating them on ice for 30 min with 300 μl cell lysis buffer. Then the collected supernatants were subjected to IP. Protein concentration was determined by the method of Micro BCA (Pierce, USA).

2.5. Dot blot experiments

Immunoprecipitated samples were analyzed by dot blot experiments. Two micro-liters of samples (IP of synthetic phospho-peptide or HL-1 cell lysate) were spotted directly onto the nitrocellulose membrane and then the dots were dried at room temperature. Membranes were blocked by soaking in TBST