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Serine^{496} of β_2 subunit of L-type Ca^{2+} channel participates in molecular crosstalk between activation of (Na^++K^+)-ATPase and the channel

Kai Y. Xu [a,*], Weizhong Zhu [b], Rui-Ping Xiao [b]

[a] Department of Surgery, Division of Cardiac Surgery, University of Maryland School of Medicine, Baltimore, MD 21201, USA
[b] Laboratory of Cardiovascular Science, National Institute On Aging, 5600 Nathan Shock, Baltimore, MD 21224, USA

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ABSTRACT

Activation of (Na^++K^+)-ATPase (NKA) regulates cardiac L-type Ca^{2+} channel (LTCC) function through molecular crosstalk. The mechanism underlying NKA–LTCC crosstalk remains poorly understood. We have previously shown that activation of NKA leads to phosphorylation of LTCC α_1 Ser^{1928}. Here we investigated whether LTCC β_2 subunit is modulated by NKA activation and found that LTCC β_2 Ser^{496} is phosphorylated in response to activation of NKA. Src inhibitor PP1 and Erk1/2 inhibitor PD98059 abolish LTCC β_2 Ser^{496} phosphorylation, suggesting that NKA-mediated β_2 Ser^{496} phosphorylation is dependent of Src/Erk1/2 signaling pathway. Protein kinase G (PKG) inhibitor KT5823 failed to inhibit the phosphorylation of β_2 Ser^{496}, indicating that the NKA–LTCC crosstalk is independent of PKG activity. The results of nifedipine sensitive ^{45}Ca influx experiments suggest that phosphorylation of β_2 Ser^{496} may play a key down-regulation role in attenuating the accelerated activity of α_1 subunit of the channel. Ouabain does not cause a phosphorylation on β_2 Ser^{496}, indicating a fundamental difference between activation and inhibition of NKA-mediated biological processes. This study provides the first evidence to demonstrate that LTCC β_2 subunit is coupled with the movement of signals in the mechanism of activation of NKA-mediated crosstalk with LTCC.

1. Introduction

Enzyme (Na^++K^+)-ATPase (NKA) [1] not only catalyzes the Na^+ and K^+ ion active transport across the plasma membrane [2,3], but is also an important signal transducer that regulates Ca^{2+} signaling and cell function [4]. We have identified an activation site of NKA which resides in the H7–H8 domain on the extracellular side of the enzyme and discovered that native activity of NKA can be further accelerated when antibody–protein interaction occurs at this unique activation site of the enzyme [5]. Binding of NKA activator SSA412 antibody to the enzyme markedly promotes NKA activity, increases cardiomyocyte contraction *in vitro* [5], and generates positive inotropic effect in mouse heart *in vivo* [6].

Cardiac voltage dependent L-type Ca^{2+} channel (LTCC) is crucial to intracellular Ca^{2+} movement and muscle contractility. Studies have shown that LTCC is composed of four subunits α_1, β_2, α_2, and δ. The α_1 subunit is the primary subunit responsible for the channel function and the β_2 subunit regulates the channel activity [7]. Recently, we have found that activation of NKA, induced by activator SSA412 antibody, markedly modulates intracellular Ca^{2+} transients by communicating with LTCC in heart cells through a signaling cascade involving Src and ERK1/2 [8], but not the well-established regulators of the channel such as the adrenergic receptor system [9] or cAMP-dependent signaling [10], nor activation of PKA [11] or CaMKII [12]. Molecular communication between activation of NKA and LTCC (crosstalk) represents a new cellular Ca^{2+} signaling mechanism which is not well understood. Identification of essential amino acids of LTCC responsible for receiving signals from activation of NKA will help to understand the underlying mechanism of the NKA–LTCC crosstalk. We have previously shown that activation of NKA leads to phosphorylation of LTCC α_1 Ser^{1928} [13], indicating the involvement of LTCC α_1 subunit in the crosstalk [8]. However, the role of β_2 subunit of the channel in the mechanism of NKA–LTCC crosstalk remains an open question. The present study is designed to explore whether LTCC β_2 subunit is coupled to the movement of signals in the molecular communication between NKA and LTCC.

2. Materials and methods

2.1. Materials

Polyclonal antibody SSA412 was generated as described previously [5]. SSA412 peptide antigen (PB412) was synthesized by BioSynthesis. Inhibitors 4-amino-5-(4-methylphenyl)-7-(t-butyl)

* Corresponding author. Address: 10 South Pine Street, MSTF-434E, Baltimore, MD 21201, USA. Fax: +1 410 706 0311.
E-mail address: kxu002@umaryland.edu (K.Y. Xu).

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pyrazolo[3,4-d]pyrimidine (PP1) and 2′-amino-3′-methoxyflavone (PD98059) were from BioMol International. Protein kinase G (PKG) inhibitor KT5823 was from Fisher Scientific. The cDNA clones of wild-type (WT) LTCC α_1 and β_2, site-specific mutated (Mut) LTCC α_1 (Ser^{1928} to Ala) and β_2 (Ser^{496} to Ala) subunits, specific anti-phospho-Ser496 ($pSer^{496}$) and anti-phospho-Ser1928 ($pSer^{1928}$) were from Dr. Steven O. Marx's laboratory [13,14]. Anti-Cav1.2a and anti-β_2 antibodies were from Chemicon. Alkaline phosphatase conjugated secondary antibody and color-developing reagent were from Promega Corporation. HEK293 cells and Lipofectamine 2000 were from Invitrogen. Sprague Dawley rats were from Charles River Laboratories. The Animal Care and Use Committees of the University of Maryland School of Medicine approved animal protocols.

2.2. Isolation of rat cardiac myocyte

Rat ventricular myocytes were isolated as previously described [15].

2.3. NKA activity assay

Isolated rat myocytes were homogenized for 5 s at 14,000 rpm three times in 10 mM histidine (free base) buffer. NKA activity of each sample was determined based on the method of Kyte [16] with modifications as previously described [8]. The enzymatic activity is defined as the ouabain-sensitive hydrolysis of MgATP in the presence of 100 mM NaCl and 20 mM KCl. Samples were incubated with or without SSA412 (2 μM) in the presence or absence of denatured SSA412 (2 μM), PB412 (40 μM), or rabbit total IgG (2 μM). The reaction was initiated by adding MgATP (3 mM) in a final volume of 0.2 ml at 37 °C for 30 min and terminated by adding 0.75 ml quench solution and 0.02 ml developer. The concentration of phosphate was then determined at 700 nm using a spectrophotometer.

2.4. Activation of NKA and Western blotting

Rat myocytes were suspended in Hepes buffer (containing 137 mM NaCl, 4.9 mM KCl, 1.2 mM $MgCl_2$, 1.2 mM NaH_2PO_4, 15 mM glucose, and 20 mM Hepes at pH 7.4) with or without NKA activator SSA412 (2 μM) in the presence or absence of PP1 (10 μM), PD98059 (20 μM), KT5823 (1 and 10 μM), denatured SSA412 (2 μM), PB412 (40 μM) mixed with SSA412 (2 μM), and rabbit total IgG (2 μM) separately for 60 min at room temperature. All signaling pathway inhibitors were incubated with myocytes for 60 min prior to start the experiments of activation of NKA. Cell lysates were prepared by collecting the supernatants from the reaction samples after (i) suspension of the samples in 1X lysis buffer at 4 °C, (ii) sonication three times at 3–5 s interval on ice-water, and (iii) centrifugation at 14,000 rpm for 10 min. Following the determination of protein concentrations, samples (30 μg each) were mixed with electrophoresis sample buffer, boiled for 5 min, and loaded on a 7% SDS gel. Following electrophoresis, the samples were transferred from the SDS gel to a nitrocellulose membrane (0.45 μm). The nitrocellulose membranes were incubated overnight with different first antibodies including anti-LTCC α_1 (1:200), anti-LTCC β_2 (1:200), $pSer^{496}$ and $pSer^{1928}$ (1:1000) separately. The membranes were then washed and incubated with secondary antibody (1:7500) for 1 h. The color was then developed for visual analysis.

2.5. HEK293 cell transfection and nifedipine-sensitive ^{45}Ca influx

Mut LTCC α_1 (Ser^{1928} to Ala) and β_2 (Ser^{496} to Ala) subunits were transfected into HEK293 cells using LipofectAmine 2000 as described by Yang et al. [14]. Transfection of wild-type (WT) LTCC α_1 and β_2 subunits in HEK293 cell was used as control. ^{45}Ca influx was performed with transfected HEK293 cells in the presence and absence of NKA activator SSA412 and inhibitor ouabain with and without nifedipine in PBS buffer at pH 7.4 for 60 min at room temperature. The final concentrations of ^{45}Ca, $CaCl_2$, $MgCl_2$, SSA412, and ouabain in reaction mixture were 1 μCi, 10 μM, 2 mM, 1 μM, and 1 mM, respectively. The reaction was stopped by adding 5 mM EGTA on ice and cells were washed and spun at 4000 rpm for 2 min (×3) to remove extracellular ^{45}Ca. Net intracellular counts per minute (cpm) of ^{45}Ca for each sample was determined using a β-scintillation counter and concentration of ^{45}Ca was calculated by using the following equation: [(cpm of sample without nifedipine) − (cpm of sample with nifedipine)]/cpm of nCi × mg of total protein of the sample.

2.5.1. Statistics

All data are shown expressed as mean ± SEM. Differences between means were assessed using ANOVA and Bonferroni's post-test. $P < 0.01$ was considered statistically significant.

3. Results

3.1. Specificity of NKA activator SSA412 antibody

Specificity of SSA412 was determined by incubating SSA412 with isolated rat myocytes homogenates under different conditions as shown in Fig. 1. Catalytic activity of NKA was increased 2-fold (Fig. 1b) compared with the control without SSA412 (Fig. 1a). The capability of SSA412 to enhance native NKA activity was significantly eliminated when SSA412 was denatured (Fig. 1c) or with peptide blocker PB412 (Fig. 1d). No significant alterations of NKA activity were detected in the presence of total rabbit IgG (Fig. 1e). PB412 alone had no effect on the basal NKA activity (Fig. 1f).

3.2. Activation of NKA induces phosphorylation of LTCC β_2 Ser^{496}

Having demonstrated the specificity of SSA412, we next performed the activation of NKA on intact myocytes and Western blotting experiments to determine whether LTCC β_2 subunit phosphorylation is affected by activation of NKA. Incubation of isolated

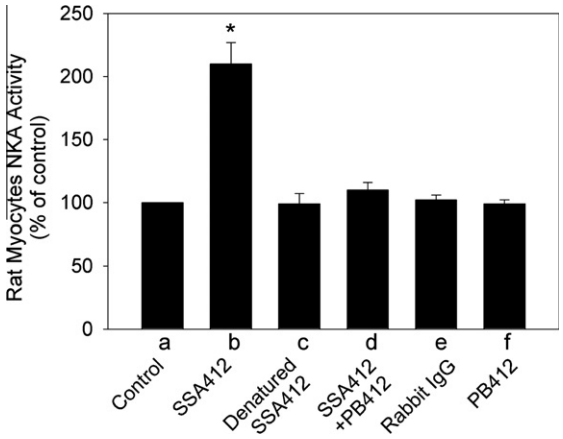

Fig. 1. Specific regulation of SSA412 on NKA. (a) Control without SSA412, (b) with active SSA412, (c) with denatured SSA412, (d) with active SSA412 + PB412, (e) with rabbit IgG, and (f) with PB412. Only binding of active SSA412 to NKA augments the catalytic activity of the enzyme. *$P < 0.01$ compared with a, c, d, e, or f. The data represent mean values of three independent experiments.