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Serine⁴⁹⁶ of β_2 Subunit of L-type Ca^{2+} Channel Participates in Molecular Crosstalk between Activation of $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$ and the Channel

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Abstract

Activation of $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$ (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 Ser¹⁹²⁸. Here we investigated whether LTCC β_2 subunit is modulated by NKA activation and found that LTCC β_2 Ser⁴⁹⁶ is phosphorylated in response to activation of NKA. Src inhibitor PP1 and Erk1/2 inhibitor PD98059 abolish LTCC β_2 Ser⁴⁹⁶ phosphorylation, suggesting that NKA-mediated β_2 Ser⁴⁹⁶ phosphorylation is dependent of Src/Erk1/2 signaling pathway. Protein kinase G (PKG) inhibitor KT5823 failed to inhibit the phosphorylation of β_2 Ser⁴⁹⁶, indicating that the NKA-LTCC crosstalk is independent of PKG activity. The results of nifedipine sensitive ⁴⁵Ca influx experiments suggest that phosphorylation of β_2 Ser⁴⁹⁶ 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 Ser⁴⁹⁶, 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.

Keywords

$(\text{Na}^+ + \text{K}^+)\text{-ATPase}$; L-type Ca^{2+} channel; enzyme activator; phosphorylation; molecular crosstalk

1. Introduction

Enzyme $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$ (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

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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 Ser¹⁹²⁸ [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)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 (Ser¹⁹²⁸ to Ala) and β_2 (Ser⁴⁹⁶ to Ala) subunits, specific anti-phospho-Ser⁴⁹⁶ (pSer⁴⁹⁶) and anti-phospho-Ser¹⁹²⁸ (pSer¹⁹²⁸) 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 sec 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 Jack 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 MgCl₂, 1.2 mM NaH₂PO₄, 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 3 times at 3–5 seconds 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 α₁ (1:200), anti-LTCC β₂ (1:200), pSer⁴⁹⁶ and pSer¹⁹²⁸ (1:1000) separately. The membranes were then washed and incubated with secondary antibody (1:7500) for 1 hour. The color was then developed for visual analysis.

2.5. HEK293 Cell Transfection and Nifedipine-Sensitive ⁴⁵Ca Influx

Mut LTCC α₁ (Ser¹⁹²⁸ to Ala) and β₂ (Ser⁴⁹⁶ to Ala) subunits were transfected into HEK293 cells using LipofectAmine 2000 as described by Yang et al [14]. Transfection of wild-type (WT) LTCC α₁ and β₂ subunits in HEK293 cell was used as control. ⁴⁵Ca 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 ⁴⁵Ca, CaCl₂, MgCl₂, 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 (x3) to remove extracellular ⁴⁵Ca. Net intracellular counts per minute (cpm) of ⁴⁵Ca for each sample was determined using a β-scintillation counter and concentration of ⁴⁵Ca was calculated by using the following equation: [(cpm of sample without nifedipine) – (cpm of sample with nifedipine)]/cpm of nCi x mg of total protein of the sample.

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 two-fold (Fig. 1-b) compared with the control without SSA412 (Fig. 1-a). The capability of SSA412 to enhance native NKA activity was significantly eliminated when SSA412 was denatured (Fig. 1-c) or with peptide blocker PB412 (Fig. 1-d). No significant alterations of NKA activity were detected in the presence of total rabbit IgG (Fig. 1-e). PB412 alone had no effect on the basal NKA activity (Fig. 1-f).

3.2 Activation of NKA Induces Phosphorylation of LTCC β₂ Ser⁴⁹⁶

Having demonstrated the specificity of SSA412, we next performed the activation of NKA on intact myocytes and Western blotting experiments to determine whether LTCC β₂

subunit phosphorylation is affected by activation of NKA. Incubation of isolated rat myocytes with SSA412 prior to cell lysis led to phosphorylation of LTCC β_2 Ser⁴⁹⁶ (Fig. 2-A, lower panel: lane 2). Phosphorylation of β_2 Ser⁴⁹⁶ was not detected in control sample without NKA activator SSA412 (Fig. 2-A, lower panel: lane 1), nor in the presence of denatured SSA412, or PB412 plus active SSA412, and with rabbit total IgG (Fig. 2-B, lower panel: lanes 1, 2 & 3).

3.3. Src and Erk1/2 Signaling Pathways Linked to Ser⁴⁹⁶ Phosphorylation

To explore the potential pathways that participate in the phosphorylation on LTCC β_2 Ser⁴⁹⁶ upon NKA activation, we next determined whether Src and Erk1/2 signaling proteins are linked to and are necessary for the phosphorylation of β_2 Ser⁴⁹⁶. Activation of NKA was performed with enzyme activator SSA412 in the presence of Src inhibitor PP1 and Erk1/2 inhibitor PD98059 separately as described previously [8]. Experimental results reveal that phosphorylation of LTCC β_2 Ser⁴⁹⁶ is abolished by PP1 and PD98059 as shown in Fig. 2-A, lanes 3 & 4 of the lower panel.

3.4. Effect of PKG Inhibitor KT5823 on the Phosphorylations of LTCC β_2 Ser⁴⁹⁶ and α_1 Ser¹⁹²⁸

PKG directly phosphorylates β_2 Ser⁴⁹⁶ in LTCC heterologously expressed HEK293 cells [14]. We determined whether PKG regulates the activation of NKA-mediated phosphorylation of LTCC. Western blotting analysis shows that KT5823 [17], a highly specific cell-permeable inhibitor of PKG, failed to eliminate the SSA412-induced phosphorylation of both β_2 Ser⁴⁹⁶ and α_1 Ser¹⁹²⁸ (Fig. 2-C) using two concentrations (1.0 and 10 μ M).

3.5. Impact of β Ser⁴⁹⁶ Phosphorylation on ⁴⁵Ca Influx through LTCC

To search the functional role of phosphorylation of β_2 Ser⁴⁹⁶ in NKA-LTCC crosstalk, WT and Mut LTCC α_1 and β_2 subunits were expressed into HEK293 cells and Western blots and nifedipine sensitive ⁴⁵Ca influxes were performed under activation and inhibition of NKA conditions. HEK293 cell was chosen for the study since it contains necessary signaling proteins including Src and Erk1/2 [18], and does not express LTCC and the Na⁺/Ca²⁺-exchanger (NCX) [19]. Thus, HEK293 cells provide a unique micro-environment for activation of NKA-mediated molecular crosstalk following expression of LTCC. In addition, absence of NCX would help in calculating the net nifedipine sensitive ⁴⁵Ca influxes through LTCC. Fig. 3A shows that WT and Mut LTCC α_1 (left upper panel lanes b & c) and β_2 (right upper panel lanes b & c) subunits were successfully transfected into HEK293 cells, whose native form does not have these LTCC subunits (lane a in both left and right upper panels). Western blots further reveal that SSA412 (Fig. 3A-left, middle panel lane b) and ouabain (Fig. 3A-left, lower panel lane b) induce phosphorylation of α_1 Ser¹⁹²⁸ on WT LTCC α_1 subunit (Fig. 3A, lane b in both left middle and lower panels), but not in Mut α_1 (Ser¹⁹²⁸ to Ala) transfected cells (Fig. 3A, lane c in both left middle and lower panels). In striking contrast, phosphorylation of β_2 Ser⁴⁹⁶ only occurs under activation of NKA condition by SSA412 (Fig. 3A-right, middle panel lane b), but not by ouabain through inhibition of NKA (Fig. 3A-right, lower panel lane c). The results of nifedipine sensitive ⁴⁵Ca influx experiments show that intracellular ⁴⁵Ca concentration is respectively 1.19 $\pm$ 0.18, 1.94 $\pm$ 0.16, 0.43 $\pm$ 0.12, and 0.46 $\pm$ 0.20 nCi/mg protein for transfected HEK293 cells under the condition i) WT α_1 + β_2 , ii) WT α_1 + Mut β_2 , iii) Mut α_1 + WT β_2 , and iv) Mut α_1 + β_2 in the presence of SSA412 (Fig. 3B-e, f, g, & h). Cell ⁴⁵Ca concentration was significantly enhanced without phosphorylation of β_2 Ser⁴⁹⁶ (Fig. 3B-f vs. e) in the presence of SSA412. Without phosphorylation of α_1 Ser¹⁹²⁸, ⁴⁵Ca influx was significantly reduced (Fig. 3B-g or h vs. e). No nifedipine sensitive ⁴⁵Ca influxes were detected in the absence of SSA412 or ouabain under the same experimental conditions (Fig. 3B-a, b, c, & d), indicating

the specificity of activation of NKA-induced ^{45}Ca influx. Nifedipine sensitive ^{45}Ca influx through LTCC is respectively 4.40 and 4.36 nCi/mg protein for samples of WT $\alpha_1 + \beta_2$ and Mut $\alpha_1 + \beta_2$ under ouabain-induced inhibition of NKA conditions (Fig. 3B-i & j).

4. Discussion

4.1. LTCC β_2 Serine⁴⁹⁶ Participates in Activation of NKA-Medicated Crosstalk

Phosphorylation of protein plays a significant role in signaling transduction and represents an important regulatory mechanism in cellular processes. Phosphospecific antibodies are powerful research tools, which can identify residues precisely that are modulated by cell signaling pathways. Using phospho-epitope specific antibody pSer⁴⁹⁶ [14], Western blotting analysis reveals that LTCC β_2 Ser⁴⁹⁶ is phosphorylated in an activation of NKA-dependent manner (Fig. 2), demonstrating that β_2 Ser⁴⁹⁶ participates in the movement of signals during the molecular crosstalk between NKA and LTCC. Without NKA activator SSA412, no phosphorylation of β_2 Ser⁴⁹⁶ was detected (Fig. 2-A), indicating that activation of NKA indeed triggers a signaling cascade to phosphorylate β_2 Ser⁴⁹⁶. No phosphorylation of LTCC β_2 subunit was detected in the presence of denatured SSA412, peptide blocker PB412, or rabbit total IgG, further demonstrating the specificity of activation of NKA-dependent β_2 Ser⁴⁹⁶ phosphorylation.

We have previously shown that activation of NKA phosphorylates LTCC α_1 Ser¹⁹²⁸ [8]. Our current study reveals that inhibition of NKA by ouabain also phosphorylates LTCC α_1 Ser¹⁹²⁸ (Fig. 3A-left, lower panel lane b). This finding not only suggests that LTCC α_1 Ser¹⁹²⁸ is a common phosphorylation site for both activation and inhibition of NKA to regulate the function of LTCC α_1 subunit, but also provides new evidence to support other laboratories' reports that inhibition of NKA by ouabain regulates LTCC α_1 subunit activity [20,21]. The fact that ouabain does not induce phosphorylation of β_2 Ser⁴⁹⁶ (Fig. 3A-right, lower panel lane b), demonstrates a fundamental difference between activation and inhibition of NKA in regulating LTCC activity.

4.2. Phosphorylation of β_2 Ser⁴⁹⁶ is Dependent of Src/Erk1/2 Pathway

We have previously demonstrated that activation of NKA-enhanced LTCC activity is dependent of a phosphorylation cascade involving Src and ERK1/2 and independent of Ca^{2+} , adrenergic receptors, or activation of cAMP, PKA, or CaMKII [8]. Experimental results reported here show that Src inhibitor PP1 and Erk1/2 inhibitor PD98059 abolish β_2 Ser⁴⁹⁶ phosphorylation (Fig. 2-B), indicating a functional link between the Src/Erk1/2 signaling cascade and the β_2 Ser⁴⁹⁶ phosphorylation. These data confirm the important role of Src/Erk1/2 cascade in NKA-LTCC crosstalk and demonstrate that phosphorylation of β_2 Ser⁴⁹⁶ and α_1 Ser¹⁹²⁸ shares, in part, the same signal transmission pathway. Whether they also share a same signaling protein for direct phosphorylation at two different sites of LTCC remains to be determined. It has been demonstrated that cGMP-PKG signaling pathways modulate LTCC β_2 Ser⁴⁹⁶ and α_1 Ser¹⁹²⁸ phosphorylation in HEK293 cells [14]. Our data reveal that both β_2 Ser⁴⁹⁶ and α_1 Ser¹⁹²⁸ are phosphorylated by the NKA activator SSA412 in the presence of PKG specific inhibitor KT5823 (Fig. 2-C), demonstrating that activation of NKA-mediated phosphorylation of both β_2 Ser⁴⁹⁶ and α_1 Ser¹⁹²⁸ is independent of PKG activity.

4.3. Functional Role of β_2 Ser⁴⁹⁶ Phosphorylation in NKA-LTCC Crosstalk

Our experimental results show that intracellular concentration of ^{45}Ca in WT $\alpha_1 + \beta_2$ transfected HEK293 cells (Fig. 3B-e) is approximately 2.6-fold that of Mut $\alpha_1 + \beta_2$ transfected cells (Fig. 3B-h), suggesting that phosphorylation of LTCC α_1 Ser¹⁹²⁸ plays an important role in increasing LTCC activity in the activation of NKA-induced crosstalk with

LTCC. Phosphorylation of β_2 Ser⁴⁹⁶ alone does not increase ⁴⁵Ca influx (Fig. 3B-g), further demonstrating that phosphorylation of α_1 Ser¹⁹²⁸ up regulates LTCC function. Without phosphorylation of both α_1 Ser¹⁹²⁸ and β_2 Ser⁴⁹⁶, nifedipine sensitive ⁴⁵Ca influx occurs in Mut $\alpha_1 + \beta_2$ transfected cells (Fig. 3B-h), suggesting that other phosphorylation sites of the channel may be involved to promote LTCC activity. In the absence of phosphorylation of LTCC β_2 Ser⁴⁹⁶, intracellular concentration of ⁴⁵Ca in WT $\alpha_1 + \text{Mut } \beta_2$ transfected HEK293 cells (Fig. 3B-f) is 1.6- and 4.2-fold that of WT $\alpha_1 + \beta_2$ transfected cells (Fig. 3B-e) and Mut $\alpha_1 + \beta_2$ transfected cells (Fig. 3B-h), respectively, suggesting that phosphorylation of β_2 Ser⁴⁹⁶ may be linked to reduction of α_1 subunit activity. This potential down-regulatory role of phosphorylation of LTCC β_2 Ser⁴⁹⁶ is further confirmed by ouabain-induced ⁴⁵Ca influx (Fig. 3B-i & j). Without phosphorylation of β_2 Ser⁴⁹⁶, ouabain-induced ⁴⁵Ca influx in WT $\alpha_1 + \beta_2$ transfected HEK293 cells (Fig. 3B-i) is approximately 3.7-fold that of the same cells in the presence of SSA412 (Fig. 3B-e). Significantly increased ⁴⁵Ca influx through LTCC in inhibition of NKA conditions by ouabain (Fig. 3B-i & j) may be, in part, due to the lack of phosphorylation of β_2 Ser⁴⁹⁶. The present study provides new evidence to suggest that phosphorylation of the LTCC β_2 subunit may be a unique and important component for activation of NKA-induced modulation of LTCC function.

4.4. Significance of Self-Regulatory Property of LTCC

Yang et al have shown that phosphorylation of β_2 Ser⁴⁹⁶ likely mediates an inhibition of the channel in HEK293 cells [14]. It has also been proposed that α_1 Ser¹⁹²⁸ mediates up-regulation of the channel activity [11,13,14,22]. Our studies demonstrate that both Ser¹⁹²⁸ and β_2 Ser⁴⁹⁶ are phosphorylated when NKA is activated (Fig. 2-C). Our previous observations show that α_1 Ser¹⁹²⁸ phosphorylation leads to up-regulation of LTCC activity[8] and current findings suggest that phosphorylation of β_2 Ser⁴⁹⁶ may lead to down-regulation of channel activity (Fig. 3B). These data are consistent with other laboratories' findings and further suggest that a self-regulatory property of the channel may be present during the crosstalk between activation of NKA and LTCC. We have previously demonstrated that activation of NKA induces positive inotropic effect [5]. The potential self-regulatory effect of phosphorylation on LTCC Ser¹⁹²⁸ and β_2 Ser⁴⁹⁶ subunits may provide a reasonable explanation for a putative cellular state that activation of NKA may induce an intracellular Ca²⁺ increase without toxic Ca²⁺ overload.

Fig. 4 shows our current model for the activation of NKA-mediated crosstalk with LTCC: interaction of SSA412 and NKA at the activation site of the enzyme triggers a signal to activate Src and Erk1/2, which then relay signals, likely indirectly, to phosphorylate LTCC α_1 Ser¹⁹²⁸ for up-regulation of the channel activity. Activation of the NKA-triggered signal also phosphorylates β_2 Ser⁴⁹⁶ for down-regulation of LTCC, presumably, by a negative feedback effect to reduce the sensitivity of the channel and attenuate LTCC α_1 activity. The involvements of additional signaling proteins and amino acid residues of the channel that might participate in the NKA-LTCC crosstalk are under investigation.

5. Conclusion

This study provides the first evidence that β_2 Ser⁴⁹⁶ is phosphorylated in response to activation of NKA, suggesting that LTCC β_2 subunit is coupled with the movement of signals in the molecular communication between activation of NKA and LTCC. Our study further suggests that phosphorylation of β_2 Ser⁴⁹⁶ may play a key down-regulation role to modulate LTCC α_1 subunit function. Inhibition of NKA by ouabain does not cause a phosphorylation on β_2 Ser⁴⁹⁶, indicating a fundamental difference in mechanism between activation and inhibition of NKA-mediated crosstalk with LTCC.

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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.

Fig. 2.

Western blotting analyses of β_2 Ser⁴⁹⁶ phosphorylation. Isolated rat myocytes were incubated with different inhibitors in the presence or absence of NKA activator SSA412, denatured SSA412, PB412, or rabbit total IgG, as indicated in the figure. Nitrocellulose membranes were immunoblotted with anti- β_2 (A, B & C: upper panels) as protein concentration controls or anti-pSer⁴⁹⁶ antibodies (A & B: lower panels; C: 2nd panel). Anti- α_1 (C: 3rd panel) and anti-pSer¹⁹²⁸ (C: 4th panel) antibodies were used to monitor LTCC α_1 subunit and phosphorylated Ser¹⁹²⁸, respectively. Phosphorylations of Ser⁴⁹⁶ and Ser¹⁹²⁸ are detected in the presence of PKG inhibitor KT5823. Src inhibitor PP1 and Erk1/2 inhibitor PD98059 significantly inhibit β_2 Ser⁴⁹⁶ phosphorylation.

Fig. 3.

A) Expression and phosphorylation of LTCC α_1 and β_2 subunits in HEK293 cells under different activation and inhibition of NKA conditions. WT and Mut LTCC α_1 and β_2 subunits were expressed into HEK293 cells separately. Specific antibodies were used to determine phosphorylations of α_1 Ser¹⁹²⁸ and β_2 Ser⁴⁹⁶ in HEK293 cells following activation and inhibition of NKA. Left: upper panel, immunostaining with control anti-LTCC α_1 antibody; middle and lower panels, stained with anti-pSer¹⁹²⁸ antibody. Right: upper panel, stained with control anti-LTCC β_2 antibody; middle and lower panels, stained with anti-pSer⁴⁹⁶. The data show that both SSA412 and ouabain induce phosphorylation of Ser¹⁹²⁸, but only activation of NKA induces phosphorylation of Ser⁴⁹⁶. **B)** Nifedipine sensitive ⁴⁵Ca influx. LTCC-expressed HEK293 cells were incubated with 1 μ Ci ⁴⁵Ca and 10 μ M CaCl₂ for 60 min at RT in the presence and absence of 1 μ M SSA412 or 1 mM ouabain with and without nifedipine. a) WT LTCC α_1 and β_2 , b) WT α_1 + Mut β_2 , c) Mut α_1 and WT β_2 , d) Mut α_1 + Mut β_2 , e) a + SSA412, f) b + SSA412, g) c + SSA412, h) d + SSA412, i) a + ouabain, j) d + ouabain. All data represent mean values of three independent experiments.

$P < 0.01$: Data compared with control background without SSA412 or Ouabain. * $P < 0.01$: Data compared with e. \$ $P < 0.01$: Data compared with e or h. There is no significant difference between i and j.

Fig. 4.

Potential elements of PKG-independent signaling mechanism underlying NKA-LTCC crosstalk. Binding of activator SSA412 to NKA not only enhances native catalytic activity of the enzyme, but also triggers signal to activate Src and Erk1/2. These signaling proteins would then relay the signals to phosphorylate both LTCC β₂ Ser⁴⁹⁶ and α₁ Ser¹⁹²⁸. Black arrows indicate the direction of activation of NKA-induced signaling pathway. The dotted lines show undiscovered proteins that may participate in the activation of NKA-induced signal transduction pathway. Phosphorylation of α₁ Ser¹⁹²⁸ may lead to up-regulation of LTCC activity. Phosphorylation of β₂ Ser⁴⁹⁶ may be linked to down-regulation of the channel.