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Mechanistic Distinction Between Activation and Inhibition of (Na⁺+K⁺)-ATPase-Mediated Ca²⁺ Influx In Cardiomyocyte

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Abstract

(Na⁺+K⁺)-ATPase (NKA) mediates positive inotropy in the heart. Extensive studies have demonstrated that the reverse-mode Na⁺/Ca²⁺-exchanger (NCX) plays a critical role in enhancing intracellular Ca²⁺ concentration in the inhibition of NKA-induced positive inotropy by cardiac glycosides. Little is known about the nature of the NCX functional mode in the activation of NKA-induced positive inotropy. Here, we examined the effect of NKA activator SSA412 antibody on ⁴⁵Ca influx in isolated rat myocytes and found that KB-R7943, a NCX reverse-mode inhibitor, fails to inhibit activation of NKA-induced ⁴⁵Ca influx, suggesting that Ca²⁺ influx via reverse-mode NCX does not mediate this process. Nifedipine, an L-type Ca²⁺ channel (LTCC) inhibitor, completely blocks the activation of NKA-induced ⁴⁵Ca influx, suggesting that LTCC is responsible for the moderate increase of intracellular Ca²⁺. In contrast, inhibition of NKA by ouabain induces 4.7-fold ⁴⁵Ca influx compared with the condition of activation of NKA. Moreover, approximately 70% ouabain-induced ⁴⁵Ca influx was obstructed by KB-R7943 and only 30% was impeded by nifedipine, indicating that both LTCC and NCX contribute to the rise of intracellular Ca²⁺ and that NCX reverse-mode is the major source for ⁴⁵Ca influx induced by the inhibition of NKA. This study provides direct evidence to demonstrate that activation of NKA-induced Ca²⁺ increase is independent of reverse-mode NCX and pinpoints a mechanistic distinction between activation and inhibition of the NKA-mediated Ca²⁺ influx pathways in cardiomyocytes.

Keywords

(Na⁺+K⁺)-ATPase; Na⁺/Ca²⁺-exchanger; Ca²⁺ influx; allosteric activator; digitalis drugs

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1. Introduction

(Na⁺+K⁺)-ATPase (NKA) is the target receptor for cardiac glycoside drugs for the treatment of congestive heart failure [1–3]. This key enzyme consists of α and β subunits and only the α subunit couples the hydrolysis of ATP to countertransport 3Na⁺ and 2K⁺ across the plasma membrane [4,5]. All isoforms of NKA α subunit share the same catalytic function to maintain cell membrane potential, control cell volume and regulate cardiac contraction [6]. Extensive studies have shown that cardiac glycoside-induced positive inotropy is based on the inhibition of NKA activity and impairment of ion active transport of the enzyme. This in turn, causes a rise of intracellular Na⁺ concentration ($\uparrow$ [Na⁺]_i) and triggers the reverse mode function of NCX (Ca²⁺ influx), leading to an increase of intracellular Ca²⁺ concentration ($\uparrow$ [Ca²⁺]_i) and resulting a positive inotropic effect in the heart [2]. Under normal conditions, NCX removes 1 Ca²⁺ in exchange for 3Na⁺ (Forward-mode, Ca²⁺ efflux) and is considered one of the most important cellular mechanisms for removing Ca²⁺. However, in the presence of cardiac glycosides, the reverse-mode function of NCX is the hallmark indicator of inhibition of NKA-mediated $\uparrow$ [Ca²⁺]_i and positive inotropic effect [2].

Recently, we have identified an allosteric activation site residing on the H7-H8 domain of NKA α subunit and found that binding of allosteric activator SSA412 to this activation site of NKA not only augments the native activity of the enzyme (activation of NKA), but also induces positive inotropic effect [7,8]. To date, the mechanism underlying the activation of NKA-mediated $\uparrow$ [Ca²⁺]_i is not completely understood. Would SSA412 trigger NCX reverse-mode function to import Ca²⁺? What is the nature of NCX in the mechanism of the activation of NKA-induced $\uparrow$ [Ca²⁺]_i? The objective of this study is to answer these fundamental questions for better understanding of the mechanism of NKA activation controlled increase in cardiac contraction.

2. Materials and methods

2.1. Materials

Polyclonal antibody SSA412 was generated as described previously [7]. Nifedipine [inhibitor of L-type Ca²⁺ channel (LTCC)] [9] and KB-R7943 (inhibitor of NCX) [10] were from Tocris (Ellisville, MO). Calcium-45 at a concentration of 37.92 mCi/ml was from PerkinElmer (Boston, MA). Sprague Dawley rats were from Charles River (Germantown, MD). Dog kidney tissues were gifts from Dr. Jack Kyte's laboratory (University of California, San Diego, USA). The Animal Care and Use Committees of the University of Maryland School of Medicine approved the animal protocol.

2.2. Isolation of rat cardiac myocyte and measurement of NKA activity

Rat ventricular myocytes were isolated and cardiac NKA activity was determined as described previously [11].

2.3. Inhibitor sensitive ⁴⁵Ca influx

⁴⁵Ca influx was performed in isolated rat myocytes in the presence and absence of SSA412 with or without 10 μ M nifedipine (LTCC inhibitor) and 5 μ M KB-R7943 (NCX inhibitor) in a buffer containing 137 mM NaCl, 4.9 mM KCl, 1.2 mM MgCl₂, 1.2 mM NaH₂PO₄, and 10 mM Hepes at pH 7.4 for 60 min at room temperature. The final concentrations of ⁴⁵Ca, CaCl₂, SSA412, and ouabain in the reaction mixture were 20 nCi, 10 μ M, 0.5 μ M, and 3 mM. The reaction was stopped by adding 5 mM EGTA on ice and the cells were washed and spun at 2000 rpm for 2 min (x2) to remove extracellular ⁴⁵Ca. The total intracellular counts per minute (cpm) of ⁴⁵Ca for each sample was determined by using a β -scintillation counter and the concentration of ⁴⁵Ca was calculated as described previously [12].

Statistics- All data are expressed as mean $\pm$ SEM. Student's *t* test and paired *t* test were applied when appropriate. A *P* value less than 0.01 was considered statistically significant.

3. Results

3.1. Reverse-mode NCX does not participate in the activation of NKA-mediated $\uparrow[\text{Ca}^{2+}]_i$

We measured the NKA activator SSA412 initiated movement of Ca^{2+} from the extracellular to the intracellular compartment in isolated rat myocytes. Inhibitor sensitive ^{45}Ca influx was determined using nifedipine and KB-R7943 with or without NKA activator SSA412 or inhibitor ouabain. No significant difference of intracellular ^{45}Ca concentration ($[\text{Ca}^{45}]_i$) in the samples with 10 μM nifedipine (Fig. 1A-b) or 5 μM KB-R7943 (Fig. 1A-c) was detected compared with the control cells (Fig. 1A-a). However, binding of SSA412 to NKA α subunit caused an 86.2 ± 11 pCi ^{45}Ca influx into the cells (Fig. 1A-d). Nifedipine (10 μM) completely blocked SSA412-induced ^{45}Ca influx (Fig. 1A-e), but 86.0 ± 18 pCi ^{45}Ca was detected in the cells with 5 μM KBR7943 (Fig. 1A-f). In contrast, inhibition of NKA by ouabain induced a 402 ± 77 pCi ^{45}Ca influx (Fig. 1A-g). In the presence of 10 μM nifedipine or 5 μM KB-R7943, nifedipine-resistant ^{45}Ca influx was 274 ± 78 and 127 ± 61 pCi for KB-R7943-resistant ^{45}Ca influx (Fig. 1A-h and i). Ouabain-induced ^{45}Ca influx was completely abolished in the presence of both nifedipine and KB-R7943 (Fig. 1A-j).

As an important control experiment parallel to that shown in Fig. 1A, NKA enzymatic activity was determined for all samples under the corresponding experimental conditions, except without ^{45}Ca (Fig. 1B-a-j). The NKA activity was 100 ± 0 , 101 ± 4 , and $102 \pm 3\%$ for control cells (Fig. 1B-a), the cells with 10 μM nifedipine (Fig. 1B-b), and with 5 μM KB-R794 (Fig. 1B-c), respectively. In the presence of SSA412, NKA activity was increased to 240 ± 20 , 245 ± 15 , and $242 \pm 18\%$ (Fig. 1B-d, e, and f), compared with the control cells (Fig. 1B-a). Ouabain completely inhibited NKA activity under conditions as shown in Fig. 1B-g to j.

4. Discussion

4.1. A fundamental difference between activation and inhibition of NKA-mediated $\uparrow[\text{Ca}^{2+}]_i$

KB-R7943 primarily acts on external NCX sites and blocks the reverse-mode of NCX in intact cells [10]. Our experimental results reveal that 5 μM KB-R7943 failed to inhibit SSA412-induced ^{45}Ca influx (Fig. 1A-f), demonstrating the absence of NCX reverse-mode function during NKA activation in the myocytes. The fact that similar concentrations of $[\text{Ca}^{45}]_i$ were detected in the presence of SSA412 with (Fig. 1A-f) or without KB-R7943 (Fig. 1A-d), further indicating that NCX does not contribute to the activation of NKA-mediated $\uparrow[\text{Ca}^{2+}]_i$. Nifedipine completely blocked SSA412-induced ^{45}Ca influx (Fig. 1A-e), suggesting that LTCC bears the major responsibility for the activation of NKA-induced moderate increase of $\uparrow[\text{Ca}^{2+}]_i$. Taken together, these results suggest that NCX reverse-mode may not participate in the mechanism of activation of NKA-mediated $\uparrow[\text{Ca}^{2+}]_i$. In contrast, inhibition of NKA by ouabain induced a substantial 4.7-fold ^{45}Ca influx (Fig. 1A-g) compared with the condition of activation of NKA (Fig. 1A-d), revealing a marked difference between activator and inhibitor-induced $\uparrow[\text{Ca}^{2+}]_i$. Moreover, approximately 70% of ouabain-induced ^{45}Ca influx was obstructed by KB-R7943 (Fig. 1A-i), illustrating that the reverse-mode of NCX is the major source for $\uparrow[\text{Ca}^{2+}]_i$, which further pinpoints a fundamental difference between activation and inhibition of NKA-mediated $\uparrow[\text{Ca}^{2+}]_i$. Only 30% ouabain-induced ^{45}Ca influx was impeded by nifedipine (Fig. 1A-h), indicating that LTCC also contributes to $\uparrow[\text{Ca}^{2+}]_i$ under NKA inhibition conditions. ^{45}Ca influx was completely abolished in the presence of both nifedipine and KB-R7943 (Fig. 1A-j), further confirming that ouabain-induced ^{45}Ca influx was through both the reverse-mode NCX and

LTCC. In addition, the catalytic function of NKA was rigorously determined side by side under various experimental conditions throughout ^{45}Ca influx investigation. Significantly increased native activity of NKA induced by SSA412 (Fig. 1B, d-f) and completely inhibited activity of the enzyme by ouabain (Fig. 1B, g-j), compared with the control cells (Fig. 1B, a-c), verified the designated experimental conditions in Fig. 1A for ensuring the conclusion drawn from each result.

4.2. Distinct pathways for Ca^{2+} influx

Based on our experimental observations, Fig. 2 illustrates the distinct pathways between activation and inhibition of NKA-mediated $\uparrow[\text{Ca}^{2+}]_i$. Under normal physiological condition, NCX forward-mode removes Ca^{2+} and imports Na^+ while NKA transports Na^+ out and K^+ in across the plasma membrane. LTCC activity is also required for maintaining the Ca^{2+} balance of the cell (Fig. 2A). The binding of SSA412 to NKA accelerates enzyme turnover rate and stimulates the enzyme signaling transduction function to phosphorylate LTCC, resulting in moderate $\uparrow[\text{Ca}^{2+}]_i$ and enhanced cardiac cell contraction [11,12]. In the case of activation of NKA, the forward-mode function of NCX remains intact (Fig. 2B) and no $\uparrow[\text{Na}^+]_i$ occurred [8]. Under the condition of inhibition of NKA, ouabain abolishes NKA activity and Na^+/K^+ transport, resulting in a significant $\uparrow[\text{Na}^+]_i$, which causes the reverse-mode of NCX to become the principal pathway that loads the cell with Ca^{2+} . Along with the Ca^{2+} influx from LTCC, this combination leads to a mass $\uparrow[\text{Ca}^{2+}]_i$ and Ca^{2+} overload (Fig. 2C).

This study provides direct evidence to demonstrate that activation of NKA-induced $\uparrow[\text{Ca}^{2+}]_i$ is independent of reverse-mode NCX and pinpoints a fundamental distinction between activation and inhibition of NKA-mediated Ca^{2+} influx pathways in cardiomyocytes. It has been reported that cardiac glycosides-induced Ca^{2+} influx via NCX reverse-mode is involved in arrhythmogenic Ca^{2+} overload [13]. To date, modulating Ca^{2+} removal from the cell via NCX is one of the critical approaches to reduce arrhythmias [14] and that the absence of NCX reverse mode is of particular interest for the potential treatment of arrhythmias related to Ca^{2+} overload [14,15]. The findings of our study may potentially provide a new clinical strategy for the treatment of heart failure to avoid arrhythmia mediated by Ca^{2+} overload.

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Fig. 1.

(**A**) Inhibitor sensitive ^{45}Ca influx. Isolated rat myocytes were used for the ^{45}Ca influx experiments under various conditions. a) Control myocytes, b) a + nifedipine, c) a + KB-R7943, d) a + SSA412, e) d + nifedipine, f) d + KB-R7943, g) a + ouabain, h) g + nifedipine, i) g + KBR7943, j) g + nifedipine + KB-R7943. * $P < 0.01$: Data compared with control background a. # $P < 0.01$: Data compared with g. (**B**) NKA activity was determined for all samples under the same experimental conditions as shown in part A except without ^{45}Ca . All data represent mean $\pm$ SEM values of 4–6 independent experiments.

Fig. 2.

Schematic drawing of potential functional mode of NCX in the mechanisms of activation and inhibition of NKA-modulated Ca²⁺ movement. (A) Forward-mode NCX in normal myocytes, (B) intact forward-mode NCX under activation of NKA condition, (C) reverse-mode NCX under inhibition of NKA condition. Arrows indicate the direction of ion movement. Increased arrow width represents increased rate of ion transport.