0026-895X/09/7504-774–781
MOLECULAR PHARMACOLOGY
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*Mol Pharmacol* 75:774–781, 2009

Vol. 75, No. 4
52597/3447218
*Printed in U.S.A.*



# Activation of $(Na^+ + K^+)$-ATPase Modulates Cardiac L-Type $Ca^{2+}$ Channel Function

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Received October 8, 2008; accepted January 2, 2009

## ABSTRACT

Cellular $Ca^{2+}$ signaling underlies diverse vital biological processes, including muscle contractility, memory encoding, fertilization, cell survival, and cell death. Despite extensive studies, the fundamental control mechanisms that regulate intracellular $Ca^{2+}$ movement remain enigmatic. We have found recently that activation of the $(Na^+ + K^+)$-ATPase markedly potentiates intracellular $Ca^{2+}$ transients and contractility of rat heart cells. Little is known about the pathway responsible for the activation of the $(Na^+ + K^+)$-ATPase-initiated $Ca^{2+}$ signaling. Here, we demonstrate a novel mechanism in which activation of the $(Na^+ + K^+)$-ATPase is coupled to increased L-type $Ca^{2+}$ channel function through a signaling cascade involving Src and ERK1/2 but not well established regulators of the channel, such as adrenergic receptor system or activation of PKA or CaMKII. We have also identified $Ser^{1928}$, a phosphorylation site for the $\alpha$1 subunit of the L-type $Ca^{2+}$ channel that may participate in the activation of the $(Na^+ + K^+)$-ATPase-mediated $Ca^{2+}$ signaling. The findings reported here uncover a novel molecular cross-talk between activation of the $(Na^+ + K^+)$-ATPase and L-type $Ca^{2+}$ channel and provide new insights into $Ca^{2+}$ signaling mechanisms for deeper understanding of the nature of cellular $Ca^{2+}$ handling in heart.

$(Na^+ + K^+)$-ATPase (NKA), a key sarcolemmal membrane enzyme, regulates intracellular ion homeostasis by controlling sodium/potassium active transport in animal cells (Kyte, 1981; Shull et al., 1985; Skou, 1988; Holmgren et al., 2000). Over the past half a century, extensive studies have demonstrated that inhibition of NKA by digitalis and related cardiotonic steroids increases intracellular $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) through an elevated intracellular $Na^+$ ($[Na^+]_i$) and switches $Na^+/Ca^{2+}$ exchanger (NCX) function to the reverse mode (Adams et al., 1982; Allen et al., 1985; Smith, 1988; Bers, 2002; Altamirano et al., 2006). However, little is known about functional role of activation of NKA in intracellular $Ca^{2+}$ signaling. We have recently identified an activation site that resides within the H7-H8 domain of the $\alpha$-subunit of NKA and is distinct from the inhibitory cardiotonic steroid binding site (Xu, 2005). The turnover rate of NKA is significantly augmented when the site-specific antibody 412 (SSA412), a specific NKA activator, binds to the activation site of the enzyme (Xu, 2005). We have further demonstrated that activation of NKA by SSA412 increases myocyte $Ca^{2+}$ transient in vitro and augments mouse heart contraction in vivo, a finding naively at odds with the classic understanding of how NKA affects $Ca^{2+}$ signaling in the heart, without affecting intracellular $[Na^+]$ (Xu, 2005; Xu et al., 2006). Search for the new mechanism underlying the activation of NKA-initiated $Ca^{2+}$ signaling is the purpose of the current investigation.

## Materials and Methods

**Materials.** General reagents were purchased from Sigma Chemical unless otherwise specified. Affinity-purified polyclonal antibody

This work was supported in part by the National Institutes of Health National Heart, Lung, and Blood Institute [Grant HL52175]; by the National Institutes of Health National Institute of Arthritis and Musculoskeletal and Skin Diseases [Grant AR44197]; by the Intramural Research program of the National Institutes of Health National Institute on Aging; and by a seed grant from Johnson & Johnson.

D.I.L. and M.G.K. contributed equally to this work.

Article, publication date, and citation information can be found at http://molpharm.aspetjournals.org.

doi:10.1124/mol.108.052597.

**ABBREVIATIONS:** NKA, $(Na^+ + K^+)$-ATPase or sodium and potassium ion activated adenosinetriphosphatase (EC 3.6.1.3); NCX, $Na^+/Ca^{2+}$ exchanger; PP1, 4-amino-5-(4-methylphenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine; *N*-[2-(4-bromocinnamylamino)ethyl]-5-isoquinoline; LTCC, L-type $Ca^{2+}$ channels; BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid; PD98059, 2′-amino-3′-methoxyflavone; AR, adrenergic receptor; PKA, protein kinase A; CaM, $Ca^{2+}$/calmodulin; CaMKII, $Ca^{2+}$/calmodulin-dependent protein kinase II; CICR, $Ca^{2+}$ induced $Ca^{2+}$ release mechanism; PKC, protein kinase C; SSA412, site-specific antibody 412.

SSA412, the NKA activator, was generated in New Zealand white rabbits against the extracellular $^{897}$DVEDSYGQQWTYEQR$^{911}$ region of the rat $\alpha$-subunit of NKA. Anti-Src [pTyr$^{418}$] and PD98059 were purchased from Invitrogen (Carlsbad, CA). Anti-Src control antibody was from Santa Cruz Biotechnology Inc. (Santa Cruz, CA) Antibody AB5412, which specifically recognizes cardiac L-type $Ca^{2+}$ channels (LTCC), was from Chemicon (Billerica, MA). Anti-Erk1/2 (as control) and anti-Erk1/2 [p-Thr$^{185}$/Tyr$^{187}$] were from Cell Signaling Technology (Danvers, MA). Anti-phosphoro-Ser$^{1928}$ (pSer$^{1928}$) was a gift from Dr. Steven O. Marx (Columbia University College of Physicians and Surgeons, New York, NY) (Yang et al., 2005). Inhibitors PP1 and H89 were from BIOMOL Research Laboratories (Plymouth Meeting, PA). Western Color-developing reagent was obtained from Promega Corporation (Madison, WI). Coomassie Blue (1×) was from Bio-Rad Laboratories (Hercules, CA). Sprague-Dawley rats were purchased from Charles River Laboratories, Inc. (Wilmington, MA). The Animal Care and Use Committees of the University of Maryland School of Medicine approved animal protocols.

**SSA412 Antibody and Western Blot Analysis.** Polyclonal antibody SSA412 specifically recognizes the extracellular D–R region of the $\alpha$-subunit of NKA. Western blotting experiments were performed to determine whether SSA412 interacts with LTCC or stimulates Src and Erk1/2 activity. Rat heart cell lysates and SSA412 immunoprecipitates were prepared as described previously (Xu, 2005). For Src and Erk1/2 activation, freshly isolated rat cardiac myocytes were incubated with or without 2 $\mu$M SSA412 in the presence or absence of PP1 for 60 min at room temperature. Samples were then suspended in 2× electrophoresis sample buffer (Bio-Rad Laboratories), boiled for 5 min, and loaded on 7 or 10% SDS gels. The proteins were subsequently transferred from the SDS gel to a nitrocellulose membrane using an electroblotting apparatus. The nitrocellulose membrane was blocked with BSA for 1 h and incubated with SSA412 or other antibodies, including anti-Src [pTyr$^{418}$] (1:500) and anti-Erk1/2 [p-Thr$^{185}$/Tyr$^{187}$] (1:1000) separately overnight. The membrane was then incubated with alkaline phosphatase conjugated secondary antibody (1:7500) for 1 h and washed three times for 5 min each with Tris-buffered saline, 0.05% Tween 20, and once for 5 min with Tris-buffered saline. The color was developed using a reagent containing a mixture of NBT and BCIP (Promega) for visual analysis.

**Isolation of Cardiac Myocytes.** Rat ventricular myocytes were isolated as described previously (Hall et al., 2006), and experiments were carried out according to the guidelines of the University of Maryland School of Medicine animal welfare committees. In brief, adult male rats were anesthetized with pentobarbital sodium (50 mg/kg i.p.). Hearts were quickly excised, mounted on a Langendorff apparatus, and perfused in a retrograde fashion with buffer containing type 2 collagenase (Worthington Biochemicals, Freehold, NJ) and protease (fraction XIV; Sigma Chemical Corp., St. Louis, MO) at 37°C. Left ventricular tissue was gently minced, filtered, and washed. Dissociated cells were maintained at room temperature in HEPES-buffered Dulbecco's modified Eagle's medium supplemented with heat-inactivated fetal calf serum (10%) and insulin (1 unit/ml). All experiments were carried out within 6 h of cell isolation.

**Electrophysiology and Confocal Microscopy.** Whole-cell LTCC $Ca^{2+}$ currents were recorded using the conventional patch-clamp technique (Hamill et al., 1981). A HEKA EPC-10 amplifier was used with Patchmaster software (both from HEKA, Lambrecht/Pfalz, Germany). Patch pipettes (1.5–3 MΩ) were filled with 130 mM CsCl, 5 mM NaCl, 5 mM MgATP, 4 mM creatine phosphate, 10 mM HEPES, 1 mM $MgCl_2$, 20 mM tetraethylammonium chloride, and 4 mM BAPTA, pH adjusted to 7.2 with CsOH. Cells were superfused with a solution containing 135 mM NaCl, 5 mM KCl, 0.5 mM $MgCl_2$, 1.8 mM $CaCl_2$, 5 mM glucose, and 10 mM HEPES, pH 7.4 at 30°C. Current signals were acquired at 5 kHz and low-pass-filtered at 2.9 kHz. The holding potential was −40 mV. Linear components of leak and capacitance were removed by electronic compensation and a P/4 procedure. Series resistance was compensated to 40 to 60%. For measurements of intracellular $Ca^{2+}$, the pipette solution contained 50 $\mu$M fluo-4 and 2 mM EGTA (no BAPTA added). Myocyte fluorescence was measured with a Bio-Rad Radiance confocal scanner in line-scan mode, attached to a microscope (IX-70; Olympus, Tokyo, Japan). Image acquisition was synchronized to the delivery of voltage-clamp pulses. Analysis of current records and images was carried out using custom routines written in IDL (Research Systems).

**Measurement of Cell Contraction.** Experiments were conducted at room temperature on a Nikon TS100 inverted microscope (duBell et al., 2002), Isolated rat myocytes were suspended in buffer containing 137 mM NaCl, 5.4 mM KCl, 15 mM dextrose, 1.3 mM $MgSO_4$, 1.2 mM $NaH_2PO_4$, 1 mM $CaCl_2$, and 20 mM HEPES, pH 7.4. Cells were stimulated at 1 Hz with 1-ms pulses with a magnitude of 1.5× threshold through platinum wires mounted at the bottom of the experimental chamber. The motion of selected myocyte was continuously monitored by StepperSwitch Myocyte System (IonOptix Corporation, Milton, MA) in the presence or absence of 1 $\mu$M SSA412 with or without different inhibitors. Changes in cell length between shortening and lengthening were analyzed using IonOptix software. Contraction amplitude was indexed by the percentage shortening of cell length.

**Measurement of cAMP Accumulation.** After rat ventricle cardiomyocytes were treated with 3-isobutyl-1-methylxanthine (1 mM), a phosphodiesterase inhibitor, for 30 min at room temperature, they were incubated with either $\beta$-AR agonist isoproterenol (1 $\mu$M) for 10 min (as positive control) or SSA412 for 30 or 60 min. Cells were then harvested by centrifugation at 500*g* for 1 min and resuspended with lysis buffer (4 mM Tris-EDTA and 1 mM IBMX) at 4°C and freeze-thawed three cycles. Cell lysates (20 $\mu$l) were taken for protein concentration analyses. The samples were denatured in boiling water for 5 min and then centrifuged at 10,000*g* for 10 min. Supernatant of different samples (50 $\mu$l each) were assayed using a [$^3$H]cAMP assay kit (Amersham, Chalfont St. Giles, Buckinghamshire, UK). Protein concentration was measured by the Bradford method (Bio-Rad Laboratories) with bovine serum albumin as standard (Xiao et al., 2006).

**Determination of NKA Activity.** The ATPase assay procedure is a modification of the method of Kyte (1971) as described previously (Xu, 2005). The enzymatic activity is defined as the strophanthidin- or ouabain-sensitive hydrolysis of MgATP in the presence of 120 mM $Na^+$ and 20 mM $K^+$ with or without signaling protein inhibitor PP1 or PD98059. The reaction was initiated by adding MgATP and stopped by adding 0.75 ml of quench solution (0.5% ammonium molybdate + 0.5 M $H_2SO_4$) and 0.02 ml of developer (25 mg/ml of the mixture of 0.2 g of 1-amino-2-naphthol-4-sulfonic acid + 1.2 g of sodium bisulfate + 1.2 g of sodium sulfite) after 30-min incubation. The color was allowed to develop for 30 min at room temperature, and the phosphate was then determined at 700 nm using a spectrophotometer.

**Statistics.** Data were expressed as mean ± S.E.M. Student's *t* test and paired *t* test were applied when appropriate. A *P* value less than 0.05 was considered statistically significant.

## Results

**Activation of NKA Enhances L-Type Ca$^{2+}$ Channel Function.** Voltage dependent LTCC play a crucial role in controlling $[Ca^{2+}]_i$ dynamic balance of cardiac cells. To explore the mechanistic pathway responsible for the NKA activation-induced $Ca^{2+}$ signaling, we first determined whether LTCC is functionally linked to and regulated by the activation of NKA using the whole-cell patch-clamp technique. Specific binding of the activator SSA412 (1 $\mu$M) to NKA on the surface of isolated rat cardiomyocytes resulted in an increase of the LTCC peak currents ($I_{Ca}$), which remained steady for at least 15 min with a time-to-half-maximal response of 2.4 ± 0.4 min (Fig. 1A). Individual $I_{Ca}$ traces (test pulse, 0 mV), recorded before and 7 min after adding SSA412

antibody, showed a significant increase in the peak current and prolongation of the time course of inactivation (Fig. 1B). On average, SSA412-induced increase in $I_{Ca}$ was 1.6 ± 0.1-fold over baseline ($n$ = 8 myocytes, $p < 0.004$; Fig. 1E). Inactivation of $I_{Ca}$ as measured by the time constant ($\tau$) of the decline of current after the peak and by the full duration at half-maximum of the current transient, was significantly increased by exposure to SSA412 ($\tau$: control, 54.7 ± 7.5 ms; SSA412, 89.9 ± 14.5 ms, $p < 0.05$; FDHM: control, 43.4 ± 6.5 ms; SSA412, 92.9 ± 11.7 ms, $p < 0.05$). Binding of SSA412 to NKA enhanced $I_{Ca}$ at all voltages with a slight rightward shift of the current-voltage relationship (Fig. 1C). In contrast, heat-denatured SSA412 caused no increase in $I_{Ca}$ (Fig. 1D). No changes of LTCC current was detected when synthetic peptides were used to adsorb out the SSA412 activity (data not shown).

Cardiac LTCC is heteromultimers composed of distinct $\alpha_1$, $\beta$, and $\alpha_2\delta$ subunits. To validate whether SSA412 interacts directly with LTCC subunits, we performed immunoprecipitation followed by Western blot analysis (Fig. 1, F and G). In whole-cell lysate, SSA412 specifically recognized the $\alpha$-subunit of NKA (Fig. 1F, a) and only AB5412 interacted with the $\alpha_1$-subunit of LTCC (Fig. 1F, b). The $\alpha$-subunit of NKA was immunoprecipitated by SSA412 (Fig. 1F, c), whereas the $\alpha_1$-subunit of LTCC was not detected under the same experimental conditions (Fig. 1F, d). These data demonstrate that SSA412 does not cross-react with rat $\alpha$1-subunit of LTCC. When whole-cell lysate was stained with Coomassie Blue after the electrophoresis, Fig. 1G, a, shows the entire protein composition of rat myocytes. However, only the $\alpha$-subunit of NKA was recognized by SSA412 (Fig. 1G, b). These results further demonstrate the specificity of SSA412 and indicate that SSA412 does not interact with any subunits of LTCC.

**Activation of NKA-Induced Increase in $I_{Ca}$ Potentiates Intracellular $Ca^{2+}$ Transients.** We next characterized whether activation of NKA-provoked increase in $Ca^{2+}$ influx through LTCC serves as a signal to elevate the intracellular $Ca^{2+}$ transient and myocyte contractility (Berridge, 1993; Cheng et al., 1993; Clapham, 1995; Bers and Perez-Reyes, 1999; Bers, 2002). Using confocal line-scan imaging of fluo-4 fluorescence in conjunction with elevated EGTA to buffer free $Ca^{2+}$, we were able to steadily monitor intracellular $Ca^{2+}$ in rat ventricular myocytes. SSA412 antibody exposure resulted in an increase in intracellular $Ca^{2+}$ transients in addition to the

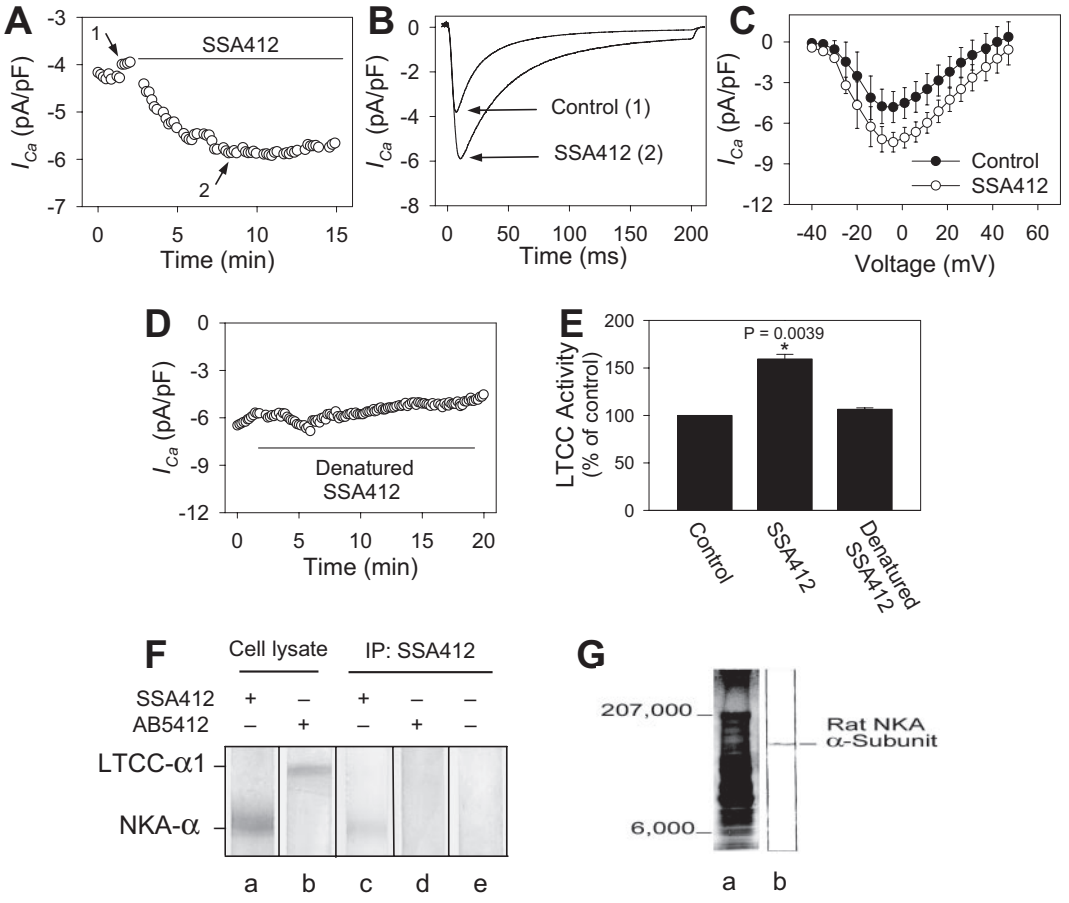


**Fig. 1.** Activation of NKA by SSA412 increases $I_{Ca}$ current in rat cardiac myocytes. A, time course of $I_{Ca}$ peak amplitude during depolarization to 0 mV before and during exposure to 1 $\mu$M SSA412. The $I_{Ca}$ was monitored every 10 s before and after adding SSA412. B, individual $I_{Ca}$ records before (control background as arrow 1 in a) and 7 min after adding SSA412 (as arrow 2 in a). C) Current-voltage relationship of $I_{Ca}$ with or without SSA412. D, time course of $I_{Ca}$ peak amplitude before and during exposure to heat-denatured SSA412. E, mean increase of $I_{Ca}$ amplitude in the presence of 1 $\mu$M SSA412. Exposure of cells to SSA412 resulted in a 60% (159.41 ± 4.95%) $I_{Ca}$ increase ($n$ = 8, $p \leq 0.0039$). No increase in cells exposed to heat-inactivated SSA412 ($n$ = 8). F, SSA412 does not cross-react with rat $\alpha$1-subunit of LTCC. Antibody AB5412 (specifically interacting with LTCC $\alpha$1 subunit) was incubated with rat heart cell lysate and immunoprecipitates with or without SSA412, during Western blotting process. a, cell lysate stained with SSA412; b, cell lysate stained with AB5412; c, immunoprecipitates stained with SSA412; d, immunoprecipitates stained with AB5412; e, secondary antibody control in immunoprecipitates. G, SSA412 does not interact with any subunits of LTCC. a, whole-cell lysate stained with Coomassie blue after electrophoresis; b, Western blot of cell lysate probed with SSA412.

augmentation of the simultaneously recorded $I_{Ca}$ (Fig. 2, A and B). Figure 2, D to F, shows the individual $I_{Ca}$, fluo-4 $Ca^{2+}$ transients, and confocal line-scan images before and 10 min after exposure to 1 $\mu$M SSA412. On average, the $Ca^{2+}$ transient amplitude was increased by 2.0 $\pm$ 0.21-fold ($n = 4$ cells, $p < 0.003$). These data suggest that activation of NKA increases $Ca^{2+}$ influx through LTCC and results in SR $Ca^{2+}$ release via $Ca^{2+}$-induced $Ca^{2+}$ release (CICR) mechanism. Ryanodine (10 $\mu$M) eliminated SR $Ca^{2+}$ release but had no effect on the SSA412-induced increase in peak $I_{Ca}$ (Fig. 2C). This result suggests that increased $I_{Ca}$ is not caused by SR $Ca^{2+}$ release, but a specific effect of activation of NKA on LTCC function in the CICR mechanism.

**Enhanced LTCC Activity by Activation of NKA Is Independent of AR/cAMP/PKA pathway or CaM/CaMKII Positive Feedback Regulation.** We have previously demonstrated that activator SSA412 specifically binds to and interacts with the activation site of NKA and does not cross-react with other proteins (Xu, 2005). Figure 1F provides additional evidence to show that SSA412 does not interact with LTCC. The fundamental question is how activation of NKA leads to a change in $I_{Ca}$. Xie and Askari (2002) have reported that NKA is a signaling transducer. We hypothesize that activation of NKA may also initiate a signaling pathway that ultimately activates LTCC. It is well known that $\alpha$- and $\beta$-adrenergic receptors ($\alpha$- and $\beta$-AR) (O-Uchi et al., 2005; Xiao et al., 2006), cAMP, protein kinase A (PKA) (Gao et al., 1997), and $Ca^{2+}$/calmodulin-dependent protein kinase II (CaMKII) (Zühlke et al., 1999; O-Uchi et al., 2005) all play important roles in the regulation of LTCC function. We next investigated whether these well established pathways participate in activation of NKA-modulated modification of LTCC function. Our results show that treatment of cells with the $\alpha$-AR blocker prazosin (Zhang et al., 2005) plus the $\beta$-AR antagonist propranolol (Sozzani et al., 1992) (7 $\mu$M each) do not affect the activation of NKA-induced positive inotropy in rat myocytes, whereas the inhibitors fully prevented the action of a $\beta$-AR agonist isoproterenol (Fig. 3A). In cardiac muscle, cAMP plays a key role in regulating both electrical and mechanical activities through the activation of the cAMP-dependent PKA (Gao et al., 1997). With respect to the downstream signaling events, we found that activation of NKA by SSA412 has no effect on cAMP formation in rat cardiac myocytes (Fig. 3B), indicating that cAMP is not involved in the NKA-dependent activation of LTCC. Moreover, a PKA inhibitor, H89 (Mueller et al., 2006), had no effect on the enhancement of $I_{Ca}$ by SSA412 (Fig. 3F), whereas it completely abolished isoproterenol-induced enhancement of $I_{Ca}$ (Fig. 3, D and E). These results further confirm that the cAMP/PKA pathway is not involved in the SSA412-evoked activation of LTCC. We further tested whether $Ca^{2+}$ ion itself would affect the cross-talk between activation of NKA and LTCC. In the absence of $Ca^{2+}$ (using 1 mM $BaCl_2$ as the extracellular charge carrier), binding of SSA412 to NKA still increased LTCC activity 72 $\pm$ 12% as shown in Fig. 3C, suggesting that activation of NKA-induced signaling pathway is independent of intracellular $Ca^{2+}$.

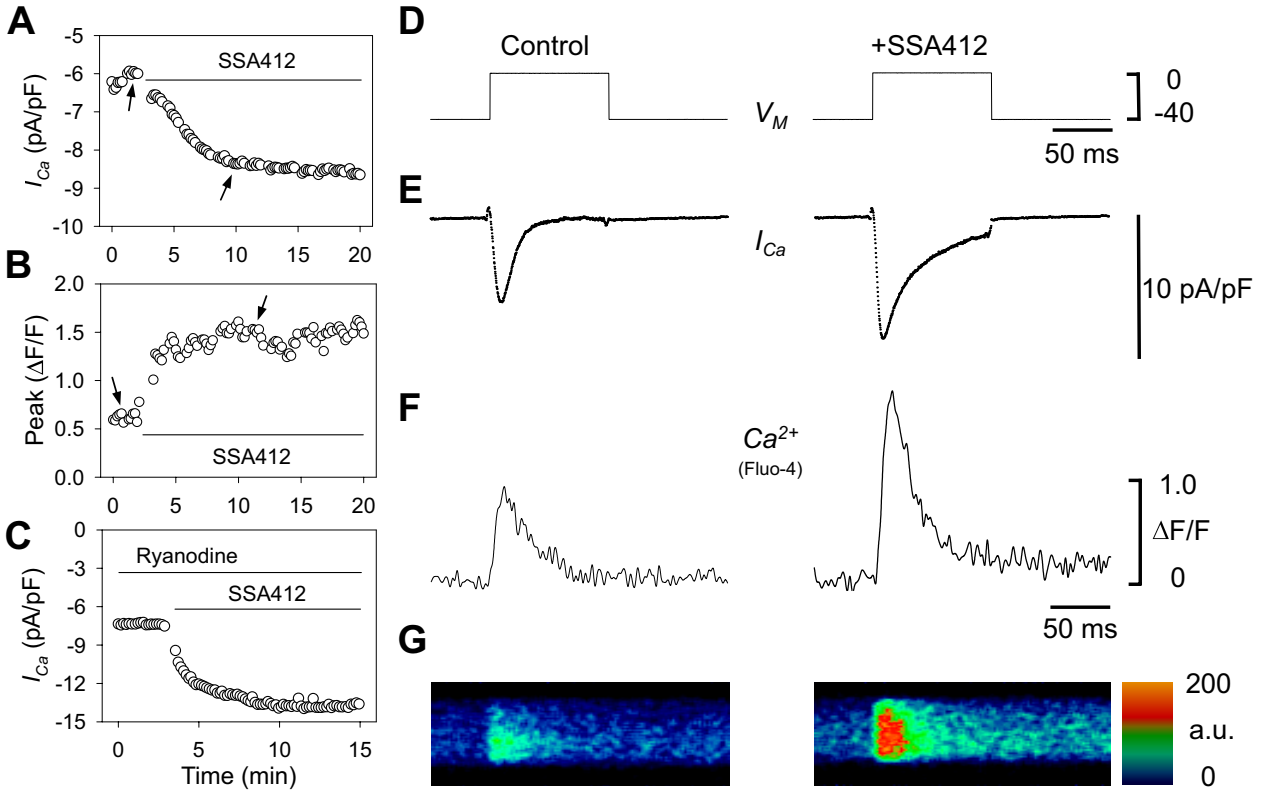


**Fig. 2.** Activation of NKA-increased $I_{Ca}$ causes increase in intracellular $Ca^{2+}$ transients. A and B, time course of simultaneously recorded increase in peak $Ca^{2+}$ current (A) and intracellular $Ca^{2+}$ transient (B) before and after exposure to SSA412 (the elevation of intracellular $Ca^{2+}$ transient in B is indexed by the fluo-4 fluorescence signal); C, time course of peak $Ca^{2+}$ current before and during exposure to SSA412 in the presence of 10 $\mu$M ryanodine; D, voltage pulse protocol; E, individual $Ca^{2+}$ current records; F, intracellular $Ca^{2+}$ transients; G, confocal line-scan images of fluo-4 fluorescence. D to G, left panel represents control; right panel indicates in the presence of SSA412. Arrows in A indicate the time of specific currents recorded as shown in B. The data represent one of four independent results.

**Role of Src and Erk1/2 in Cross-Talk between NKA and LTCC.** It has been reported that tyrosine kinase Src binds to partially inhibited NKA in the presence of ouabain (Tian et al., 2006). We next investigated whether Src plays a functional role in the cross-talk between activation of NKA and LTCC. Remarkably, a specific Src inhibitor (Dubuis et al., 2006), PP1 (10 $\mu$M), largely abolished the SSA412-induced increase in $I_{Ca}$ (Fig. 4A). Furthermore, inhibition of Erk1/2 mitogen-activated protein kinase signaling pathway by 20 $\mu$M PD98059 (Takahashi et al., 2004) also blocked the increase in SSA412-mediated LTCC (Fig. 4B). To ensure that the concentration of each inhibitor used for the experiments would not cause any nonspecific effect on NKA, PP1 and PD98059 were incubated separately with purified rat cardiac NKA under the same experimental concentrations. No inhibitory effect of 10 $\mu$M PP1 or 20 $\mu$M PD98059 on NKA activity was detected (Fig. 4C). Both Src and Erk1/2 were activated, as evidenced by their increased phosphorylation in response to the SSA412 treatment (Fig. 4D). Moreover, inhibition of Src with PP1 largely inhibited SSA412-induced activation of Erk1/2 (Fig. 4D), suggesting that the signaling pathway underlying NKA-mediated LTCC activation involves NKA, Src, Erk1/2, and LTCC sequentially.

**Ser$^{1928}$ of the $\alpha_1$-Subunit of LTCC Is Phosphorylated in Response to the Activation of NKA.** The pore-forming $\alpha_1$ subunit of LTCC contains the voltage sensor and is the major determinant of channel functional identity. Having identified that Src and Erk1/2 are necessary for the cross-talk between NKA and LTCC, we next determined a potential phosphorylation site on the $\alpha_1$-subunit of LTCC. Incubation of isolated rat myocytes with SSA412 before cell lysis led to phosphorylation of $\alpha_1$ Ser$^{1928}$ (Fig. 4E). Phosphorylation of Ser$^{1928}$ was abolished by the Src inhibitor PP1 and Erk1/2 inhibitor PD98059 (Fig. 4E), suggesting a functional link between the Src/Erk1/2 signaling cascade and the phosphorylation of the $\alpha_1$-subunit of LTCC.

## Discussion

**A New $Ca^{2+}$ Signaling Mechanism in Heart Muscle Cells.** The major finding reported here reveals that activation of NKA, induced by the specific activator SSA412, mediates cellular $Ca^{2+}$ signaling by enhancing LTCC function through a Src/Erk1/2 signaling cascade and phosphorylation of $\alpha_1$ Ser$^{1928}$ in heart cells. Binding of activator SSA412 to the activation site of NKA not only accelerates the enzyme

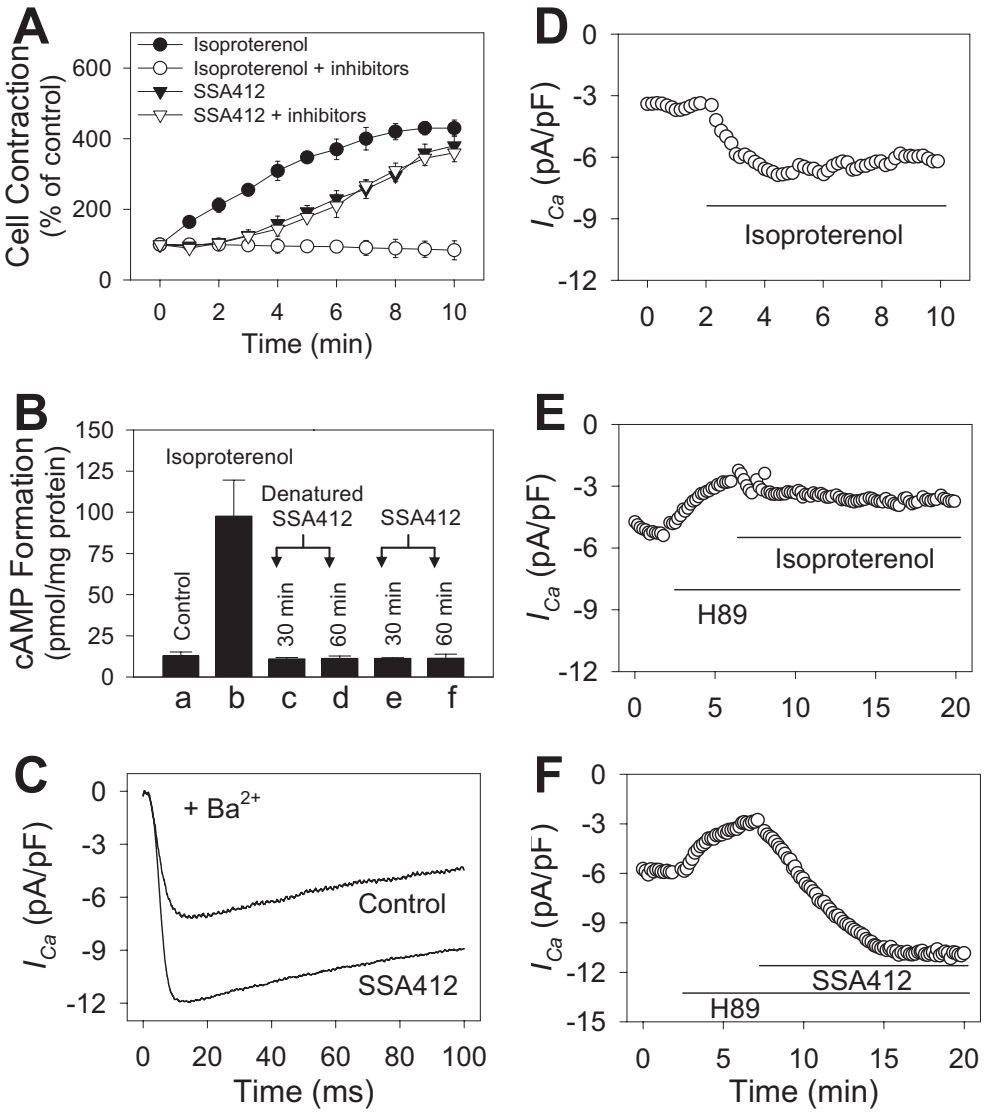


**Fig. 3.** Activation of NKA-enhanced $I_{Ca}$ is independent of $Ca^{2+}$ and adrenergic receptors, cAMP, PKA, CaM, and CaMKII signaling pathways. A, $\alpha$-AR inhibitor prazosin and $\beta$-AR inhibitor propranolol (7 $\mu$M each) did not affect activation of NKA-induced positive inotropy in rat myocytes, whereas the inhibitors eliminated the action of isoproterenol. B, activation of NKA had no effect on cAMP formation in rat cardiac myocytes. Formation of cAMP was assayed using [$^3$H]cAMP assay kit obtained from Amersham. a, control; b, with $\beta$-AR agonist, isoproterenol (1 mM) for 5 min as a positive control; c and d, cells with denatured SSA412 for 30 or 60 min; e and f, cells with SSA412 under same conditions as in c and d. C, LTCC current in the presence of 1 mM $BaCl_2$ with or without SSA412 ($n$ = 4). D to F, effect of H89 (PKA inhibitor) on $I_{Ca}$ in the presence of SSA412. Isolated rat myocytes were incubated with 2 $\mu$M H89 for 5 min before adding SSA412. $I_{Ca}$ was monitored with or without isoproterenol or SSA412 in the presence of H89. D, $I_{Ca}$ before and after adding 1 $\mu$M isoproterenol. E, $I_{Ca}$ by isoproterenol in the presence of H89. $I_{Ca}$ enhanced by isoproterenol was abolished by H89. F, H89 has no effect on the enhancement of $I_{Ca}$ induced by activation of NKA through binding of SSA412 to the enzyme. The data represent either the mean of six independent experiments (A and B) or one of the four similar studies (C through F).

catalytic activity, as we reported previously (Xu, 2005), but also initiates signaling property of NKA, leading to the positive regulation of LTCC function. Heat-denatured SSA412 caused no increase in $I_{Ca}$ (Fig. 1D), indicating that SSA412 binding to the activation site of NKA and activating of the enzyme are essential controls for the activation of NKA-enhanced LTCC activity (Fig. 1). Western blots reveal that SSA412 recognizes NKA but not subunits of LTCC (Fig. 1, F and G), demonstrating that activation of NKA-induced enhancement of $I_{Ca}$ is not a direct effect of SSA412 on LTCC. The results of whole-cell patch clamp uncovered a remarkable action that activation of NKA, induced by activator SSA412 (Xu, 2005), influences the entire population of LTCC function on an isolated rat myocyte (Fig. 1). These results provide first evidence to demonstrate that activation of NKA-initiated $Ca^{2+}$ signaling is functionally linked to LTCC.

In cardiac myocytes, the CICR mechanism is operated between LTCC and SR $Ca^{2+}$ release channels (ryanodine receptors, RyRs). Compelling evidences have demonstrated that $Ca^{2+}$ influx via LTCC can trigger SR Ca release (Endo et al., 1970; Fabiato and Fabiato, 1979; Wier, 1990; Sham et al., 1995). Ryanodine (10 $\mu$M) blocks ryanodine receptor-mediated SR $Ca^{2+}$ release and CICR in either condition with or without SSA412. Our results reveal that denatured SSA412 does not affect $I_{Ca}$ (Fig. 1D) and that no increase of $I_{Ca}$ was detected in the presence of ryanodine before the administration of SSA412 (Fig. 2C) in isolated cardiac myocytes under our experimental conditions. These data suggest that a spontaneous $Ca^{2+}$ release from SR (Fig. 2, B, F, and G) is likely to be triggered with the increase of LTCC $I_{Ca}$-induced by SSA412 (Fig. 2, A and E) and is consistent with the well established CICR principle.

LTCC is the predominant ion channel in the heart and plays an essential role in cardiac excitation and contraction. It has been demonstrated that phosphorylation of the intracellular domain of the cardiac LTCC is the critical mechanism for modulating its current. We have identified that $\alpha_1$ Ser$^{1928}$ of LTCC is phosphorylated in response to the activation of NKA. This experimental result provides direct evidence to suggest that the $\alpha_1$ Ser$^{1928}$ may play a unique role in

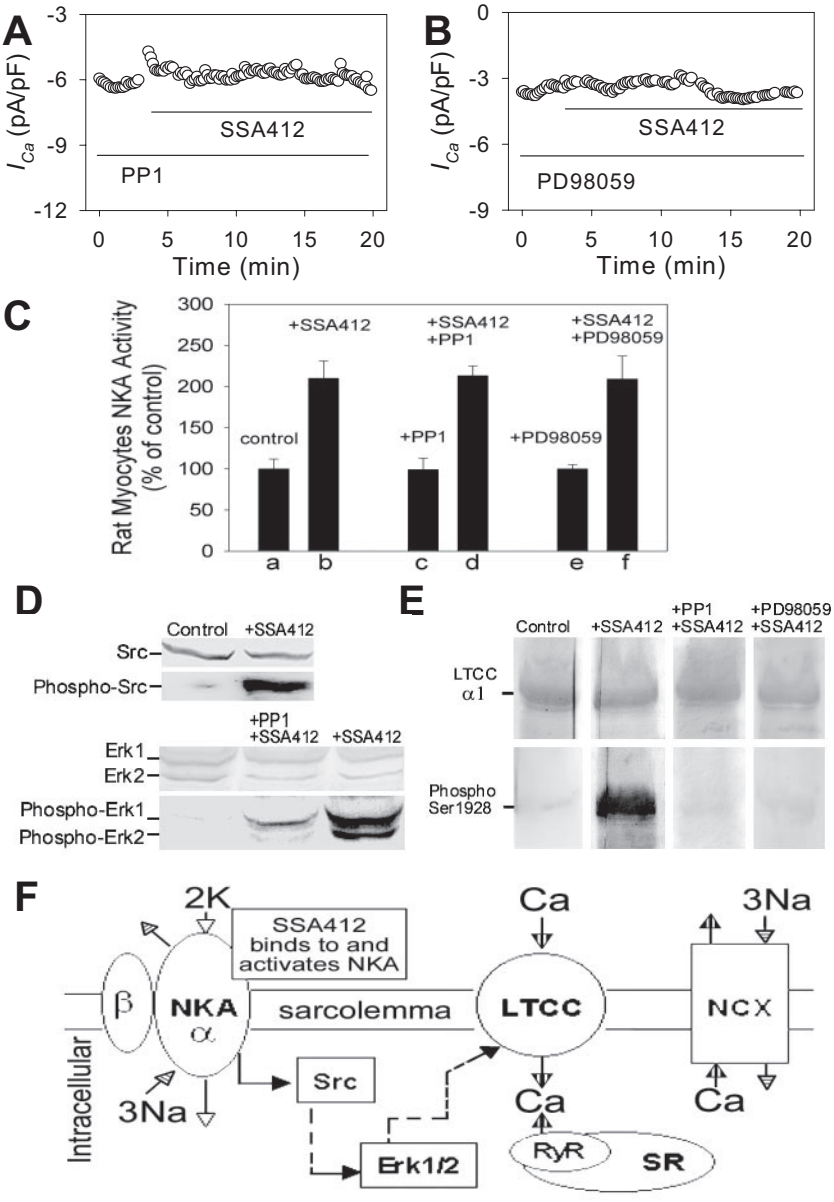


**Fig. 4.** Activation of NKA-enhanced $I_{Ca}$ is dependent on Src and Erk1/2 function. Isolated rat myocytes were incubated with different inhibitors for 1 h at room temperature before the measurements were begun. The $I_{Ca}$ was monitored before and after adding SSA412 in the continued presence of different inhibitors. A, with 10 $\mu$M Src inhibitor PP1. B, with 20 $\mu$M Erk1/2 inhibitor PD98059. Note that the enhancement of $I_{Ca}$ by exposure to SSA412 was abolished by PP1 or PD98059. C, concentration of inhibitors used in A and B above did not affect the purified rat myocyte NKA activity and activation of NKA by SSA412: a, rat myocyte NKA control; b, with SSA412; c, with PP1; d, with PP1 + SSA412; e, with PD98059; f, with PD98059 + SSA412. D, Western blotting analysis: Src phosphorylation detected by anti-Src-pTyr$^{418}$ (upper group); Erk1/2 phosphorylation detected by anti-Erk-pThr$^{185}$/Tyr$^{187}$ (lower group). E, phosphorylation of $\alpha_1$Ser$^{1928}$. The $\alpha_1$-subunit of LTCC was detected by AB5412 in cell lysate with or without SSA412 in the presence or absence of inhibitors (upper panel). Phosphorylation of $\alpha_1$Ser$^{1928}$ detected by anti-pSer$^{1928}$ (bottom middle). Src inhibitor PP1 and Erk1/2 inhibitor PD98059 significantly inhibited phosphorylation of $\alpha_1$Ser$^{1928}$ (bottom right). The data represent one of the six similar studies. F, potential molecular mechanism underlying activation of NKA-initiated signaling pathway that modulates LTCC function and $Ca^{2+}$ transport in rat ventricular myocytes. Black arrows indicate direction of activation of NKA-induced signaling pathway. Black, vertically striped, and horizontally striped arrows represent the potential $Ca^{2+}$, $Na^+$, and $K^+$ movements, respectively, in the presence of SSA412. The dotted lines imply those undiscovered proteins that may participate in the activation of NKA-induced signal transduction pathway.

receiving signals from the Src/Erk1/2 cascade to affect LTCC activity. More detailed investigation on multiphosphorylation sites of LTCC during the cross-talk between NKA and LTCC will increase our understanding of the mechanism underlying activation of NKA-mediated $Ca^{2+}$ signaling. It has been demonstrated that LTCC $\alpha_1$-$Ser^{1928}$ is a common site for LTCC $\alpha_1$-subunit phosphorylation by PKA, PKC, and PKG (Gao et al., 1997; Yang et al., 2005, 2007). Our experimental results provide additional data indicating that activation of NKA also converges on $Ser^{1928}$ of LTCC to mediate phosphorylation-dependent regulation of $Ca^{2+}$ influx.

The newly discovered molecular pathway that may be potentially responsible for the activation of NKA-mediated cellular $Ca^{2+}$ movement is illustrated in Fig. 4F. First, it is initiated by the activation of NKA through an antibody-protein interaction at the activation site of the enzyme. Second, activated NKA induces the signal to activate Src kinase. Third, Src kinase transfers the signal to Erk1/2, which leads to the phosphorylation of $\alpha_1$ $Ser^{1928}$ and subsequently the activation of LTCC. Fourth, $Ca^{2+}$ influx through activated LTCC, which simultaneously stimulates SR $Ca^{2+}$ release. Last, increased $Ca^{2+}$ may soon be up-taken back to SR by SR $Ca^{2+}$-ATPase and/or transported out of the cell via NCX standard function to reach an apparent cellular $Ca^{2+}$ ion dynamic homeostasis.

In cardiac myocytes, Activation of NKA might result in a small change of the membrane potential. However, hyperpolarization of the cell usually has a dual effect on the $Ca^{2+}$ flux via LTCC. On one hand, shifting of the membrane potential to the more negative values may decreases open probability of the LTCC; on the other hand, hyperpolarization increases driving force for calcium ions thus potentiating calcium flux. Under the physiological conditions, hyperpolarization induced by NKA activation in cardiac myocytes may be counteracted by activation of the hyperpolarization-activated cyclic nucleotide-gated channels (Arinsburg et al., 2006). It is noteworthy that activation of the cardiac hyperpolarization-activated cyclic nucleotide is potentiated by Src, which is also involved in activation of NKA-mediated signaling (Fig. 4). Considering all the above, overall effect of the NKA activation on the membrane potential as well as the effect on $Ca^{2+}$ flux via voltage-depended mechanisms is likely to be minimal.

**Mechanism of the Activation of NKA-Induced Positive Inotropy.** Intracellular $Ca^{2+}$ movement is crucial to cardiac contractility. In cardiac muscle cells, $Ca^{2+}$ entry through LTCC stimulates SR $Ca^{2+}$ release channel known as ryanodine receptor via CICR at each beat (Berridge, 1993; Cheng et al., 1993; Clapham, 1995; Bers and Perez-Reyes, 1999; Bers, 2002). We have previously reported that activation of NKA induces positive inotropy in both isolated rat myocytes in vitro and in mouse heart in vivo (Xu, 2005; Xu et al., 2006). By uncovering the fact that activation of NKA augments SR $Ca^{2+}$ release and intracellular $Ca^{2+}$ transients via enhanced LTCC and by establishing a new mechanism that activation of NKA-induced positive inotropic effect is attributable to increased $Ca^{2+}$ influx through LTCC and the resultant SR $Ca^{2+}$ release via the CICR, this study provides direct evidence for a new understanding of fundamental principles of NKA activation-induced positive inotropic effect. We have found previously that activation of NKA-induced positive inotropic effect does not alter intracellular $Na^+$ homeostasis and is independent of increasing $[Na^+]_i$ (Xu et al., 2006). Moreover, the NCX current as measured by slow voltage-ramp pulses (Hobai et al., 1997) was unchanged in the presence of SSA412 (data not shown). These results suggest that NCX is not responsible for activation of NKA-increased intracellular $[Ca^{2+}]$. However, NCX may be critically involved in quickly releasing the increased intracellular $Ca^{2+}$ in the presence of NKA activator SSA412 for cell-tolerated increases in $[Ca^{2+}]$ and dynamic cellular $Ca^{2+}$ homeostasis, because no arrhythmia was detected during the activation of NKA-mediated cardiac contraction at the cell and animal levels (Xu, 2005; Xu et al., 2006). In contrast, digitalis and related cardiac glycoside-induced positive inotropic effect is dependent on the inhibition of NKA, increasing $[Na^+]_i$, and a reversal function of NCX to increase intracellular $Ca^{2+}$. This makes a fundamental difference in mechanism between activation and inhibition of NKA-induced positive inotropy.

**Cross-Talk between NKA and LTCC Is Dependent on Src/Erk1/2 Cascade and Independent of $Ca^{2+}$.** Xie and Askari have demonstrated that partial inhibition of NKA by digitalis induces signaling transduction process between enzyme and signaling proteins (Mohammadi et al., 2001; Xie and Askari, 2002; Tian et al., 2006). It is noteworthy that our experimental results also show that activation of NKA activates both Src and Erk1/2 (Fig. 4D), suggesting that NKA may be able to act as a signaling transducer under either an activation or an inhibition condition. Both the Src inhibitor PP1 and the Erk1/2 inhibitor PD98059 significantly inhibited SSA412-enhanced $I_{Ca}$ (Fig. 4, A and B), indicating that Src and Erk1/2 are required for and essentially involved in the molecular communication of NKA and LTCC. Furthermore, inactivation of Src by PP1 largely inhibited SSA412-induced activation of Erk1/2, suggesting that ERK1/2 activation is mediated by Src activation. Dubuis et al. (2006) have reported that Src has multiple binding sites on the $\alpha$1c subunit of LTCC. Because the Erk1/2 inhibitor PD98059 significantly reduced the activation of NKA-enhanced LTCC current (Fig. 4B), it is unlikely that Src would directly phosphorylate LTCC in the presence of SSA412 but might do so through activation of Erk1/2 (Fig. 4E). We anticipate that more signaling proteins may participate in the molecular communication between activation of NKA and LTCC. It is noteworthy that Src and Erk1/2 also participate in the ouabain-induced signaling pathway (Xie and Askari, 2002), implying that activation and inhibition of NKA may share some of the similar signaling proteins in the NKA-initiated signaling transduction pathways to regulate different cellular mechanisms for $Ca^{2+}$ signaling. Le Grand et al. (1990) reported that low concentration ($<10^{-7}$ M) of ouabain stimulates LTCC currents in guinea pig cardiac myocytes, suggesting that ouabain may have a dual effect that attributes to different mechanisms depending on its different concentrations. More detailed investigations will help to better understand SSA412 and ouabain shared properties in inducing signaling cascades through NKA.

The binding of SSA412 to NKA enhances $I_{Ca}$ when $Ba^{2+}$ was used as the charge carrier (Fig. 3C), which indicates that activation of NKA-initiated protein signaling pathway is $Ca^{2+}$-independent. In contrast, the ouabain-induced PKC translocation and ERK1/2 activation are dependent on the presence of $Ca^{2+}$ (Mohammadi et al., 2001). This suggests a distinct difference in principle between inhibition and acti-

vation of NKA modulated molecular signaling processes. Because the activation of PKC, CaM, and CaMKII pathways are $Ca^{2+}$-dependent (Zühlke et al., 1999; O-Uchi et al., 2005), it is unlikely that PKC, CaM, and CaMKII would participate in the molecular communication between NKA and LTCC. Thus, adrenergic receptors, cAMP/PKA, PKC, and $Ca^{2+}$/CaM/CaMKII-mediated signaling pathways may not be essential to the modulation of LTCC by activation of NKA.

The present findings demonstrate that activation of NKA modulates LTCC function, leading to an elevated SR $Ca^{2+}$ release and intracellular $Ca^{2+}$ transients. Our data reveal a new mechanism to illustrate the basic principle of activation of NKA-evoked $Ca^{2+}$ signaling that is fundamentally different from the traditional understanding of the inhibition of NKA-induced $Ca^{2+}$ movement. Moreover, we have shown that the Src/Erk1/2 signaling cascade and $\alpha_1$ $Ser^{1928}$ are necessary components responsible for the molecular communications between activation of NKA and LTCC. These findings provide novel insights for better understanding of the nature of cellular $Ca^{2+}$ signaling and bear important clinical implications.

**Acknowledgments**

We are grateful to Drs. T. Rogers and D. Yue for important support and advices; J. Lederer and P. Cheng for helpful suggestions; L.-S. Song, I. Dick, and X. Tang for valuable technical assistance; and M. Palmer for preparing rat myocytes. We thank Dr. S. Marx for generously providing $pSer^{1928}$ antibody for the investigation.

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