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Echinochrome A regulates phosphorylation of phospholamban Ser16 and Thr17 suppressing cardiac SERCA2A Ca^{2+} reuptake

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Abstract Echinochrome A (Ech A), a marine bio-product isolated from sea urchin eggs, is known to have cardioprotective effects through its strong antioxidant and ATP-sparing capabilities. However, the effects of Ech A on cardiac excitation–contraction (E-C) are not known. In this study, we investigated the effects of Ech A on cardiac contractility and Ca^{2+} handling in the rat heart. In ex vivo Langendorff hearts, Ech A (3 μM) decreased left ventricular developing pressure to 77.7±6.5 % of basal level. In isolated ventricular myocytes, Ech A reduced the fractional cell shortening from 3.4 % at baseline to 2.1 %. Ech A increased both diastolic and peak systolic intracellular Ca^{2+} ($[Ca^{2+}]_i$). However, the ratio of peak $[Ca]_i$ to resting $[Ca]_i$ was significantly decreased. Ech A did not affect the L-type Ca^{2+} current. Inhibiting the Na^+/Ca^{2+} exchanger with either $NiCl_2$ or SEA400 did not affect the Ech A-dependent changes in Ca^{2+} handling. Our data demonstrate that treatment with Ech A results in a significant reduction in the phosphorylation of phospholamban at both serine 16 and threonine 17 leading to a significant inhibition of SR Ca^{2+}-ATPase 2A (SERCA2A) and subsequent reduced Ca^{2+} uptake into the intracellular Ca^{2+} store. Taken together, our data show that Ech A negatively regulates cardiac contractility by inhibiting SERCA2A activity, which leads to a reduction in internal Ca^{2+} stores.

Keywords Echinochrome A · Negative inotropic effect · SERCA2A inhibition · Phospholamban phosphorylation

Introduction

Echinochrome A (Ech A), 6-ethyl-2,3,5,7,8-pentahydroxy-1,4-naphthoquinone, is the lipophilic and membrane permeable dark-red pigment in sea urchin shells and spines (Fig. 1a) [2, 37]. In both animal experiments and clinical trials, Ech A showed significant cardioprotective effects against ischemia/reperfusion (IR) injury [6, 34, 3]. Histochrome, a water-soluble form of Ech A, was recently developed to treat cardiovascular diseases. It was found to reduce the size of myocardial infarction (MI) by 45 % [3] and the incidence of ventricular arrhythmias while preventing cardiac necrosis [6]. These cardioprotective properties are believed to result from its strong antioxidant activity and iron-chelating capacity [2]. Furthermore, recent data have also suggested that histochrome may have an ATP-sparing effect in the myocardium of ischemic patients [1].

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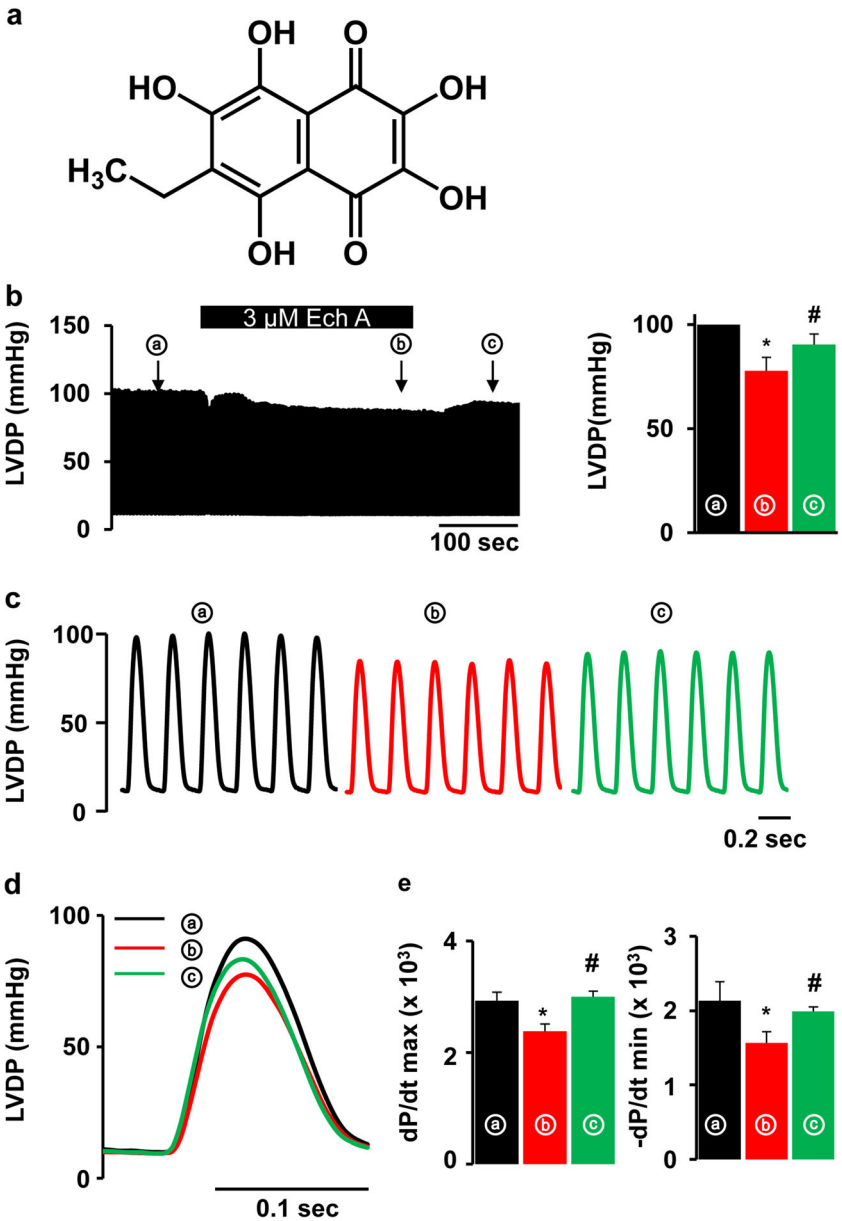

Fig. 1 Echinochrome A (*Ech A*) induces a negative inotropic effect in ex vivo hearts. Rat hearts were perfused with normal tyrode solution (NT) for a basal (ⓐ) and washout period (ⓒ) or with NT with Ech A (3 μM) (ⓑ) on a Langendorff system. LVDP and heart rate were continuously measured by a PowerLab system. **a** The chemical structure of echinochrome A (Ech A), 6-ethyl-2,3,5,7,8-pentahydroxy-1,4-naphthoquinone (FW= 266.2). **b** Representative LV pressure trace in rat heart. Basal LV pressure was adjusted to 100.3±3.2 mmHg. Ech A reduced systolic LV pressure by 22 % (77.7±6.5 mmHg) within 5 min of treatment. **c** Representative LVDP trace in selected points. **d** Superimposed LVDP trace of basal (ⓐ), Ech A (ⓑ), and washout periods (ⓒ). **e** Histogram of max or min dP/dt. Ech A reduced both dP/dt max and min (*p<0.05 vs. basal, #p<0.05 vs. Ech A. n=3). ⓐ basal, ⓑ Ech A treatment, and ⓒ washout

Because intracellular Ca^{2+} overload plays critical role in determining MI size and further functional restoration of the heart in IR injury or metabolic inhibition [28, 20], the Ca^{2+} handling proteins of the cardiac excitation–contraction (E-C) coupling machinery have been proposed to be therapeutic targets to promote cardioprotection. The major of Ca^{2+} influx occurs via the L-type Ca^{2+} channel which triggers sufficient intracellular Ca^{2+} release from the sarcoplasmic reticulum (SR) and initiates cardiac contraction. For relaxation to occur, Ca^{2+} is taken back up into the SR via SR Ca^{2+}-ATPase 2A (SERCA2A) or extruded into the extracellular space through the Na^{+}/Ca^{2+} exchanger 1 (NCX1). Interestingly, each of these Ca^{2+} handling components have been demonstrated to be involved in Ca-dependent cardiac cell death during IR injury [29, 31, 8, 39, 23, 5, 35]. Interestingly, various negative inotropic agents, such as verapamil, nifedipine or lacidipine, have shown to be cardioprotective during IR injury, likely reflecting their effects on Ca^{2+} handling [29, 5]. NCX1 knock-out or inhibition by KB-R7943 reduced the infarct size in IR injury, whereas NCX1 overexpression increases the susceptibility of heart to IR injury [8, 9]. Similarly, SERCA2A inhibition by cyclopiazonic acid or thapsigargin before ischemia or during reperfusion protected mechanical function in the ischemia/reperfusion (I/R)-treated heart [13].

Despite of the importance of intracellular Ca^{2+} regulation in both physiological and pathological conditions, there is no study investigating the impact of Ech A in cardiac E-C coupling and Ca^{2+} handling. Therefore, we investigated the impact of Ech A on E-C coupling and Ca^{2+} handling in intact rat heart and isolated cardiomyocytes. This study demonstrates,

for the first time, that Ech A is a negative inotropic drug that decreases cardiac contractility by reducing Ser16 and Thr17 phosphorylation of phospholamban leading to SERCA2A inhibition.

Materials and methods

Chemicals

SEA0400, an NCX activity inhibitor, was purchased from Chemscene (NJ, USA). Ca^{2+} indicators, cell-permeable Fluo4-AM and cell-impermeable Fura-2, were purchased from Invitrogen (Oregon, USA). All antibodies including anti-phosphor-phospholamban (Thr17 and Ser16), total phospholamban, SERCA2A, phosphor-Ca^{2+}-calmodulin-dependent protein kinase II (p-CaMKII), and β-tubulin were purchased from Santa Cruz, Inc. (Dallas, TX, USA). We used Histochrome® containing 0.02 or 1 % echinochrome A (PN002363/02) produced by Pacific Institute of Bioorganic Chemistry, Far East Branch of the Russian Academy of Sciences. All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Animals

Eight-week-old (body weight 250 g), male Wistar rats were obtained from Orient Bio, Inc. (Seongnam, Gyeonggi-do, Korea) and used for all experiments. All experimental procedures were approved by the Institutional Review Board of Animals, Inje University College of Medicine (approval number: 2013-052).

Measurement of cardiac performance

Hearts from rats were perfused with normal tyrode (NT) buffer for stabilization. Next, hearts were perfused with Ech A (3 μM) in NT for 5 min and washed by perfusing NT without Ech A again at 37 °C for 10 min [24]. A latex balloon connected to a pressure transducer was inserted into the left ventricle through the left atrium. Left ventricular (LV) pressure, heart rate, and maximum and minimum dP/dt were continuously recorded with a PowerLab system (AD Instruments, Mountain View, CA).

Isolation of single rat cardiomyocytes

Single rat cardiomyocytes were prepared as previously described [24]. Rats were anesthetized by intraperitoneal (i.p.) injection of sodium pentobarbital (100 mg/kg) and heparin (300 IU/mL/kg). The heart was rapidly excised and then perfused in a Langendorff system with NT (95 % O_2 and 5 % CO_2) at 37 °C for 10 min for stabilization. After the stabilization period, hearts were perfused with Ca^{2+}-free NT solution for 7 min, followed by Ca^{2+}-free NT solution plus 0.01 % collagenase (Yakult, Japan) for 9–13 min. Hearts were washed in oxygenated Krebs–Henseleit buffer (KB) containing (in mM) 118.5 NaCl, 4.7 KCl, 1.2 $MgSO_4$, 1.8 $CaCl_2$, 24.8 $NaHCO_3$, 1.2 KH_2PO_4, and 10 glucose for 10 min. The atria were discarded, and the left ventricular wall and septum were cut into small pieces and agitated in KB solution to dissociate cardiomyocytes.

Ca^{2+} transient and contractility measurements in field-stimulated myocytes

Isolated single cardiac myocytes were incubated with 5 μM of Fluo4-AM for 30 min and then embedded in a perfusion chamber with an electronic stimulator (IonOptix, Inc., USA). The cells were perfused with NT or various experimental solutions: Ech A (3 μM), $NiCl_2$ (10 mM), $NiCl_2$ with Ech A, SEA0400 (1 μM), SEA with Ech A, thapsigargin (TG, 100 nM), and thapsigargin with Ech A. CaT and cell contraction were elicited by field stimulation at 1 Hz and 10 V. Cell shortening was recorded by MyoCam-S and analyzed by IonWizard v 6.3 (IonOptix LLC, MA, USA). CaT in stimulated cardiac myocyte was measured by confocal microscopy in line scan mode, LSM 700 (Carl Zeiss, Germany), as previously described [17]. Briefly, Fluo4-AM-stained myocytes were excited by 488 nm, and emission signals over 505 nm were collected. Images of line scanning were acquired at sampling rate of 1.54 ms/line. The base Ca^{2+} signal (F_0) was measured during resting state without stimulation. CaT (F_1) was recorded in continuously stimulated single cardiac myocyte (electric stimulation 1 Hz, 10 V). The diastolic Ca^{2+} level in NT was considered to be 100 %. The systolic Ca^{2+} level (%) was expressed as a relative level to the diastolic Ca^{2+} level. Transient amplitude was calculated as the systolic Ca^{2+} level minus the diastolic Ca^{2+} level during each period. The rate constant of the decay of the [Ca^{2+}] was determined as a single-exponential fit of the decay phase of Ca^{2+} transient using Origin 8.0 software [38]. Intracellular Ca^{2+} modulation and cell size changes in the resting state were also measured in Fluo4-AM-stained single cardiac myocytes by confocal microscopy. All experiments were performed at room temperature.

Patch clamp recording

L-type Ca^{2+} currents were recorded from isolated rat ventricular myocytes placed onto the microscopic chamber by using Axopatch 200B amplifier (Axon Instrument) at room temperature (23±1 °C). NT was perfused during seal formation, and it contained (in mM) 143 NaCl, 5.4 KCl, 0.5 $MgCl_2$, 1.8 $CaCl_2$, 5.5 glucose, and 5 *N*-[2-hydroxyethyl]piperazine-*N*-[2-ethanesulfonic acid] (HEPES), pH was adjusted to 7.4.

For recording of L-type Ca^{2+} currents, the bathing solution was switched from NT to a solution which contained the following (in mM): 120 NaCl, 5 KCl, 1 $MgCl_2$, 1.8 $CaCl_2$, 5.5 glucose, 20 tetraethylammonium chloride (TEA-Cl), and 10 HEPES, titrated to pH 7.4 with 1 M NaOH [24]. Pipette filling solution contained (in mM) 120 CsCl, 5 NaCl, 3 $MgCl_2$, 5 MgATP, 5 ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), and 10 HEPES, titrated to pH 7.2 with 1 M CsOH. Pipettes were pulled from thin-walled borosilicate capillaries (Clark Electromedical Instruments, Pangbourne, UK) using a vertical type puller (PP-83, Narishige, Tokyo, Japan). Seals were achieved by negative pressure on to the membrane patch and were always over 2 GΩ. Only whole-cell configurations, with a series resistance less than 5 MΩ, were selected for recording L-type Ca^{2+} currents. Recordings were performed at least 5 min after whole-cell configuration to allow full dialysis of cells with the pipette filling solution. Cells were briefly (200 ms) depolarized to a potential of −40 mV from a holding potential of −80 mV in order to inactivate voltage-gated Na^+ channels. Cells were depolarized to a test potential of +10 mV (500 ms) to measure the Ca^{2+} current.

The liquid-junction potentials between bathing and pipette filling solution were calculated based on ionic mobility. The current signals were filtered at 5 kHz by a built-in four-pole Bessel type low-pass filter. Cell capacitance (pF) was automatically calculated by PatchPro based on time domain technique [26], which was used to calculate current density.

SERCA2A activity assay

The effect of Ech A on the SERCA2A activity was assessed using LV homogenate following modified methods from previous studies [14, 22]. Dissected LV samples were diluted 1:11 (wt./vol.) in ice-cold homogenizing buffer (pH 7.5) containing (in mM) 250 sucrose, 5 HEPES, 0.1 PMSF, and 0.2 % sodium azide (NaN_3). The LV was homogenized by a glass-Teflon-coupled homogenizer. To remove nuclei and heavy debris, the homogenates were centrifuged at 1,000×*g* for 10 min at 4 °C. We quantified protein concentration of the homogenates by the bicinchoninic acid assay (BCA) method. Finally, 200 μg/well of protein was used for the assay. The SERCA assay solution contained the following (in mM): 0.05 K_2-EGTA, 5 Na_2-ATP, 10 phosphocreatine, 25 HEPES, 100 KCl, and 5.5 MgCl, pH 7.0. In addition, Fura-2 (10 μM) to probe Ca^{2+}, oxalate (10 μM) to stabilize intra-SR [Ca^{2+}], and ruthenium red (3 μM) to inhibit SR Ca^{2+} efflux and mitochondrial Ca^{2+} buffering were added. Assay solutions (150 μL) were transferred to a 96-well microplate with LV homogenates. Ech A (3 μM in PBS as final concentration) or the same volume of PBS was added into each well and incubated for 5 min at room temperature. Ca^{2+}-free basal fluorescence of Fura-2 was measured by fluorescence mode of SpectraMax M2 microplate reader (Molecular Devices, Sunnyvale, CA, USA) by measuring 510-nm emission after exciting with 340-nm wavelength. Ca^{2+} was rapidly increased to 100 μM, and the subsequent decline was interpreted as SR Ca^{2+} uptake, as other Ca^{2+} removal processes were physically or pharmacologically removed. Basal (F_0) indicated Fura-2 intensity at 100 μM [Ca^{2+}]. All experiments were performed at room temperature.

Myocardial infarction assay

The cardioprotective effects of Ech A against ischemia/reperfusion-induced MI were tested on a Langendorff system. Isolated hearts (n=3 in each group) were stabilized by perfusion for 10 min with NT solution. Ischemia/reperfusion was accomplished by stopping flow for 30 min and reperfusion of the heart for 1 h. Hearts were perfused with Ech A (3 μM), TG (100 nM), or TG with Ech A solution 5 min before the ischemic periods, respectively [13]. After the reperfusion period, the heart was sliced into 2-mm tissue slices and then incubated in 1 % 2,3,5-triphenyl-2H-tetrazolium chloride (TTC) PBS solution, pH 7.4, at 37 °C for 20 min. Tissues were fixed in 10 % PBS-buffered formalin overnight at 2–8 °C. TTC-stained tissue slices were scanned by a flatbed scanner (Umax PowerLook 1100; Fremont, CA, USA), and MI size was assessed using ImageJ software (http://rsb.info.nih.gov/ij/, 1.39u, NIH, USA).

Western blot analysis

Isolated hearts were perfused with NT or Ech A (3 μM) solutions for 10 min on a Langendorff system. Atria and right ventricles were removed, and then, LV tissue was lysed in lysis buffer (20 mM HEPES [pH 7.5], 150 mM NaCl, 1 mM EDTA, 2 mM EGTA, 1 % Triton X-100, and 10 % glycerol) containing protease and phosphatase inhibitor cocktail. LV lysates were centrifuged at 10,000×*g* for 15 min at 4 °C. Protein concentrations were determined by the BCA method (Bio-Rad, USA), and 30 μg of protein was loaded per lane onto 10 % sodium dodecyl sulfate polyacrylamide gels. Gels were transferred onto nitrocellulose membranes (Whatman, Germany) and incubated with specific antibodies including SERCA2A (100 kDa), p-phospholamban Ser16 (p-PLBs16, 25 kDa) and Thr17 (p-PLBt17, 27 kDa), phospholamban (PLB, 25 kDa), and p-CaMKII (50 kDa). β-tubulin (55 kDa) was used for the internal standard protein for quantification. The relative level of each protein was quantified by MultiGauge version 2.2 (Fuji Photo Film, Japan).

Data analysis

Data were analyzed using Student's *t* test and ANOVA with Tukey's post hoc comparison of means using Origin 8.0

software (OriginLab, Northampton, MA, USA). $P<0.05$ was considered statistically significant. All data represent mean± standard error of the mean (SEM) of three independent experiments.

Results

Ech A exerts a negative inotropic effect on the ex vivo heart

The effective physiological dose of Ech A was determined to be 3 μM in our previous study [19]. First, we tested whether Ech A altered cardiac contractility and heart rate in the normal ex vivo condition by using a Langendorff perfusion system and measuring LV intraventricular pressure. Ech A treatment led to a gradual decrease in LV developing pressure (LVDP) (Fig. 1b), which was as low as 78 % of basal level. The selected LVDP trace in basal NT perfusion (a), Ech A perfusion (b), and washout with NT (c) shows that Ech A reduced LVDP peak pressure and heart rate (Fig. 1c). In addition, Ech A treatment significantly decreased the velocity to LV contraction (dP/dt max) and relaxation (dP/dt min) in perfused heart (Fig. 1d, e). The basal mean LV dP/dt max was 2,932 mmHg/s. Ech A treatment decrease of mean LV dP/dt max to 2,385 mmHg/s (19 % decrease from basal). Ech A treatment slightly reduced heart rate to 96 % of basal level (Supplementary Fig. 1).

All negative inotropic effects of Ech A were initiated within 5 min of perfusion, and these effects were reversibly washed out of Ech A by NT (<5 min). Importantly, Ech A did not cause arrhythmia during perfusion or after washout period in all treated hearts.

Ech A exerts a negative inotropic effect on isolated LV cardiac myocyte

To test whether Ech A also cause negative inotropic effects in single cardiac myocyte, we examined the effect of Ech A on single cell contraction in isolated rat LV cardiac myocytes. Isolated LV myocytes were continuously stimulated by an electrical field stimulator (1 Hz, 10 V). As under whole heart conditions, Ech A treatment rapidly reduced cell shortening (Fig. 2a). The basal mean contraction ratio was 3.4 % of cell length. Ech A treatment limited contraction to just 2.1 % of cell length (Fig. 2b). Moreover, the maximum velocity (Vmax) of contraction and relaxation was also delayed by Ech A treatment. The basal Vmax of contraction and relaxation was 169.7 and 155.6 μm/s, respectively. Ech A slowed those to 121.5 and 106.2 μm/s (Fig. 2c), respectively. The effects of Ech A were observed within 3 min of perfusion and were completely reversible by NT washout (3 min).

Ech A reduced intracellular Ca^{2+} transients

Because intracellular Ca^{2+} mobilization is necessary for cardiac E-C coupling, we tested whether Ech A treatment altered Ca^{2+} handling and resulted in a negative inotropic effect in the heart. Isolated cardiac myocytes were incubated with Fluo4-AM (5 μM, 1 h at 37 °C), a cell-permeable Ca^{2+} fluorescent indicator. The fluorescence intensity was measured by line scan mode confocal microscopy during 1-Hz electrical field stimulation. We tested the interfering effect of Ech A on the fluorescence of Fura-2 and Fluo4-AM. As results, Ech A did not alter fluorescence intensity of both Fura-2 and Fluo-4 AM in the presence of zero or 100 μM Ca^{2+} in cell-free solution (Supplementary Fig. 2). Ech A simultaneously increased both diastolic and systolic Ca^{2+} levels to 120 and 106 % of baseline, respectively, within 3 min of perfusion (Fig. 3a, b). However, the basal-to-peak amplitude of the Ca^{2+} transient was decreased, as result of a relatively higher increase of diastolic Ca^{2+} than systolic Ca^{2+} resulting from Ech A treatment (Fig. 3c). Ca^{2+} traces of each experimental period show that Ech A treatment significantly delayed intracellular Ca^{2+} decay time, suggesting inhibition of Ca^{2+} removal mechanisms (Fig. 3d). The times of decay at 50 % (t50) and 90 % (t90) were 0.19 and 0.57 s at baseline. Ech A treatment significantly prolonged the t50 and t90 to 0.29 (149 % to NT) and 0.68 s (117 % to NT), respectively (Fig. 4e). The effect of Ech A was reversible by washout with NT (<3 min). Interestingly, Ech A treatment significantly increased intracellular resting Ca^{2+} level (110 % to basal level) in non-stimulated cardiomyocytes. This increase in resting Ca^{2+} likely explains the observed decreased cell size in these myocytes to 93 % of basal condition (Supplementary Fig. 3). However, this increase in baseline cytosolic Ca^{2+} did not induce hypercontracture of cardiac myocytes.

To investigate whether the observed effects on Ca^{2+} modulation may be part of a more common response to antioxidant compounds, we tested how Ca dynamics in rat cardiomyocytes changed in response to the widely used antioxidant, *N*-acetylcysteine (NAC, 2 mM) [36]. In contrast to Ech A, the NAC treatment did not alter systolic, diastolic CaT, or time of decay compared to NT perfusion (Supplementary Fig. 4). These data indicate that the effect of Ech A on contractility is likely independent of its general antioxidant properties.

Sequestration of Ca^{2+} during diastole is mainly facilitated by Ca^{2+} reuptake into the SR through SERCA and also by extrusion through the NCX [21]. Therefore, we next tested whether Ech A may have an inhibitory effect on NCX or SERCA activity.

Ech A did not change L-type Ca^{2+} channel or NCX activity

Since the L-type Ca^{2+} channel (LTCC) is a central component in E-C coupling, we directly tested the effect of Ech A on the activity of LTCC by using the patch clamp technique. Test

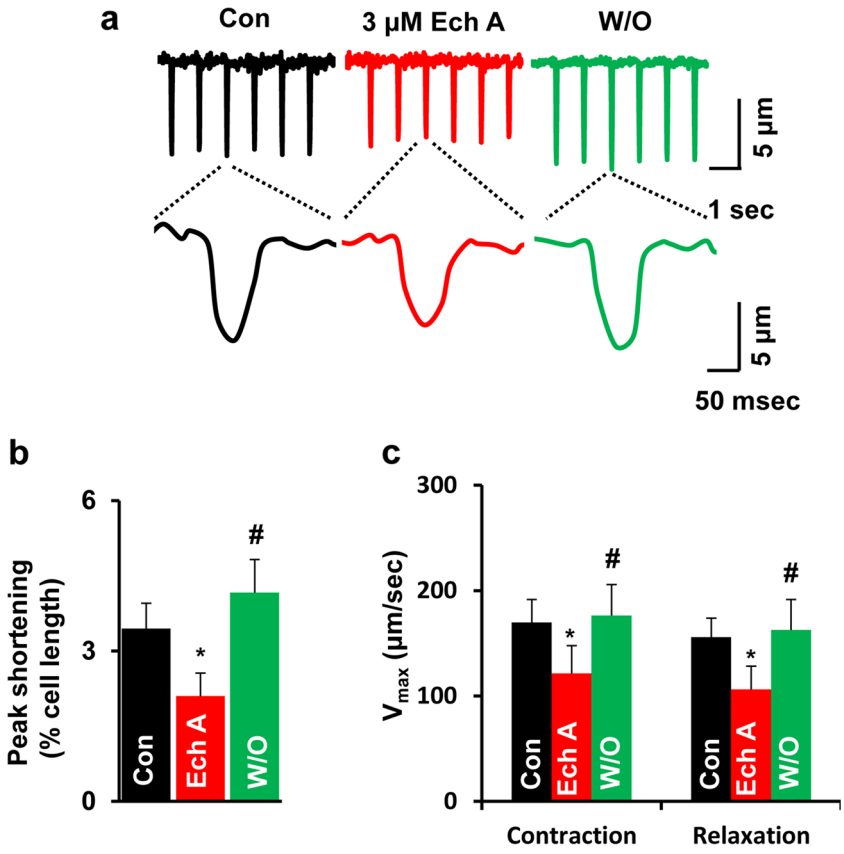

Fig. 2 Ech A treatment reduced cardiac contractility and shortened cell length in an isolated cardiac myocyte. **a** Representative trace of single cell contraction in NT control (*Con*, 5 min), Ech A (3 μM/L, 5 min), and washout (*W/O*, 10 min) periods (*top*). Representative single contraction trace in each period (*bottom*). The cell was continuously stimulated by a field stimulator (1 Hz, 10 V). **b** Peak shortening (% cell length) comparison between Con, Ech A, and W/O periods. **c** Comparison of maximal velocity of cell contraction and relaxation (*$p<0.05$ vs. Con, #$p<0.05$ vs. Ech A. $n=8$)

pulses were applied every 5 s, and the effects of Ech A were tested by comparing peak amplitudes of LTCC before and after Ech A (Fig. 4a, b). Ech A did not alter the time dependent decay of LTCC current density in rat cardiac myocytes (Fig. 4c). Even though Ech A did not affect the amplitude of LTCC at a test pulse, there is a possibility that Ech A elongated and shifted the current–voltage relationship either to the right or to the left to let amplitude at a test pulse of +10 mV unchanged. Therefore, currents were obtained by applying test potentials ranging from −45 to +50 mV in 5 mV step (Fig. 4d), and current–voltage relationships were constructed and compared (Fig. 4e). As shown in Fig. 4e, there was no significant change in the current–voltage relationship by Ech A.

As a next step, CaTs in the presence of NCX inhibitors, SEA0400 and $NiCl_2$, were assessed to evaluate the effect of Ech A on NCX activity [4]. Treatment with NCX inhibitor, SEA0400 (1 μM), did not change diastolic Ca^{2+} levels but reduced systolic Ca^{2+} levels in single cardiomyocytes, whereas Ech A elevated diastolic Ca^{2+} levels in the presence of SEA0400, resulting in a decreased amplitude of CaT (Fig. 5). SEA400 treatment did not significantly modulate the time of Ca^{2+} decay, but simultaneous treatment with SEA400 and Ech A significantly delayed both the t50 and t90 compared to NT (a) or SEA400 (b) treatment (Fig. 5e). In addition, we observed no additive effect of SEA400 with Ech A compared with treatment with Ech A alone, on the slowing of the time of decay (Ech A vs. SEA/Ech A, t50 149±5 vs. 143±7 %, t90 117±6 vs. 128±9 %, respectively). $NiCl_2$ (10 mM) treatment remarkably reduced CaT in isolated rat LV cardiac myocytes and blocked the electrically stimulated intracellular Ca^{2+} peaks. Importantly, when in the presence of $NiCl_2$, Ech A still elevated Ca^{2+} level compared to $NiCl_2$ treatment alone (Supplementary Fig. 5a, b). This suggests that the Ni^{2+}-sensitive NCX activity is not the direct target of Ech A. Unlike treating with Ech A alone, the effect of treating with $NiCl_2$, together with Ech A, was not fully reversible after washout with NT (Supplementary Fig. 5d). Moreover, arrhythmic Ca^{2+} oscillations appeared in the washout period (Supplementary Fig. 5c). These results indicate that the L-type Ca^{2+} channel and NCX were not likely targets of Ech A.

Ech A inhibits SERCA 2A Ca^{2+} uptake by modulation of PLB phosphorylation

Next, we assessed SERCA 2A Ca^{2+} uptake ratio in SR-containing LV homogenates. Figure 6 shows Ca^{2+} uptake traces of control and Ech A-treated SR-containing homogenates. Ech A (3 μM) treatment significantly delayed the speed of Ca^{2+} uptake by 55 %. This indicates an inhibitory effect of Ech A on SERCA 2A Ca^{2+} uptake activity (Fig. 6a, b).

SERCA2A activity is regulated by the protein phospholamban. The inhibitory effect phospholamban exerted on

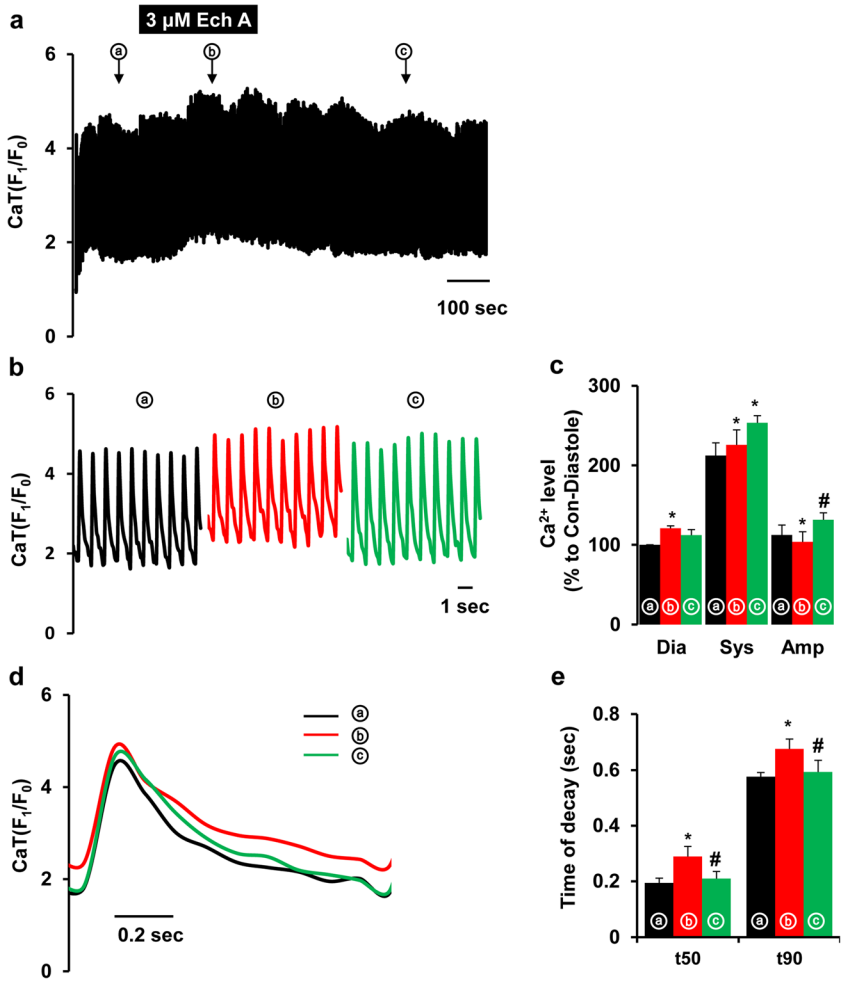

Fig. 3 Ech A treatment reduced Ca^{2+} transient (*CaT*) and delayed time of Ca^{2+} decay. **a** Representative CaT in stimulated cardiac myocyte during NT control (ⓐ, 3 min), Ech A (ⓑ, 5 min), and washout (ⓒ, 7 min) periods. CaT was measured in Fluo4-AM-stained single cardiac myocyte by using confocal microscopy in the line scan mode. Base Ca^{2+} signal (F_0) was measured during the resting state without stimulation. CaT (F_1) was recorded in continuously stimulated single cardiac myocyte (electric stimulation 1 Hz, 10 V). **b** Representative CaT of Con, Ech A, and W/O periods. Ech A significantly elevated the diastolic Ca^{2+} level while also slightly elevating the level of systolic Ca^{2+}. **c** Diastolic (*Dia*), systolic (*Sys*), and Ca^{2+} amplitude (*Amp*) comparison between Con, Ech A, and W/O periods. The diastolic Ca^{2+} level of Con period was considered as 100 %. Systolic Ca^{2+} level (%) is expressed as the increase over the Con diastolic Ca^{2+} level. Amplitude (*Amp*) was calculated as systolic Ca^{2+} level subtract diastolic Ca^{2+} level in each period. **d** Superimposed single trace of a CaT at basal (ⓐ), Ech A (ⓑ), and W/O (ⓒ). **e** CaT decay time at 50 and 90 %. The rate constant of decay of $[Ca^{2+}]$ determined from single-exponential fits to the decay phase of the $[Ca^{2+}]$. (*$p<0.05$ vs. Con, #$p<0.05$ vs. Ech A. $n=5$)

SERCA2A is relieved upon phosphorylation of the protein at either Ser16 or Thr17. Therefore, we compared protein expression levels of SERCA2A, p-PLBs16, and p-PLBt17, and total PLB, PKA, and p-CaMKII in control or Ech A-treated hearts. Ech A (3 μM, 10 min) perfusion significantly reduced phosphorylation of PLB at Ser16 (40 % of control) and Thr17 (59 % of control) sites. Protein expression levels of SERCA2A, total PLB, catalytic subunit of PKA α/β, and p-CaMKII were similar in control and Ech A-treated hearts which suggested that reduced phosphorylation of PLB was not mediated by PKA or CaMKII signaling pathway (Fig. 6c. d). We further tested whether Ech A treatment enhanced dephosphorylation of PLB by protein phosphatase 1 (PP1). Protein inhibitor −1 (I-1) is the main inhibitor of PP1. Phosphorylation of I-1 at Thr35 activates I-1 activity, while phosphorylation at Ser67 inhibits it [33, 7, 32]. Ech A treatment reduced phosphorylation of Thr35 site of I-1. These results suggested that SERCA2A activity is a direct target of Ech A through its enhanced effect on PLB dephosphorylation.

Ech A attenuates MI in ischemia/reperfused heart

To test whether the inhibitory effect of Ech A on SERCA is protective against I/R injury, MI sizes in I/R-treated hearts were measured in control, Ech A, TG, and TG/Ech A combination pretreated hearts ($n=3$ in each group). Pretreatment for 5 min with 3 μM Ech A before global ischemia significantly reduced MI size after reperfusion (Fig. 7). The infarct size in

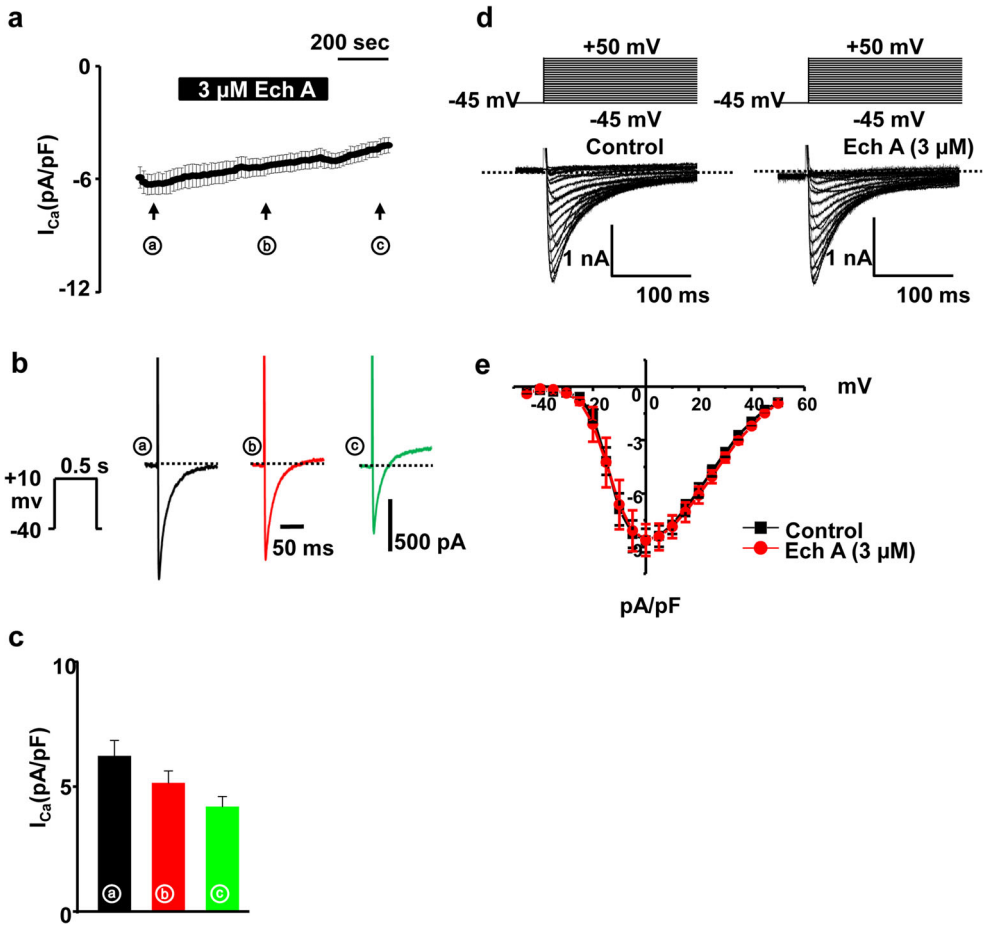

Fig. 4 Ech A treatment does not alter L-type Ca^{2+} current. Currents were induced by a test potential to +10 mV from a pretest potential of −40 mV (**a**–**c**) or by series of test potentials ranging between −45 and + 50 mV (**d**–**e**). **a** Effects of Ech A on averaged L-type Ca^{2+} current density (I_{Ca}) (n=4). **b** Representative n-type Ca^{2+} currents before (ⓐ), during (ⓑ), and after (ⓒ) Ech A (3 μM) treatment (*see text in more detail*). **c** I_{Ca} comparison in each period. **d** Currents induced by a series of test potentials from −45 to + 50 mV in 5-mV step. *Dashed lines* denote zero current level. **e** Reconstructed peak current density–voltage relationships of control (n=9) and Ech A (n=10). Membrane capacitance (pF) was used to calculate current density (pA/pF)

control was 31±3.6 % of total area of sliced hearts, and Ech A treatment significantly reduced the MI size to 12.2±4.6 % of the hearts. We compared the cardioprotective effect of Ech A with pretreatment with TG alone and co-pretreatment with Ech A and TG in I/R-treated hearts. Both TG and TG/Ech A pretreatment significantly reduced I/R-induced MI sizes in all treated hearts in comparison with non-treated control (vs. Con p<0.05) (Fig. 7). We observed no differences in MI size between treatments (Ech A vs. TG vs. TG/Ech A group. p= n.s.). In addition, the effects of Ech A on CaT dynamics, including the increase of diastolic Ca^{2+} and decrease of diastolic to systolic Ca^{2+} amplitude, were similar when compared with those in the TG- or TG and Ech A-treated hearts. These data indicate that co-treatment did provide any additive effects in comparison with single treatment of TG or Ech A (Supplementary Fig. 6).

Discussion

The cardioprotective effect of Ech A observed in previous studies has been suggested to result from its ability to act as a strong ROS scavenger [1, 34, 6]. In the present study, for the first time, we demonstrate that Ech A is able to modulate Ca^{2+} handling. Specifically, we found that Ech A exerts a negative inotropic effect in the heart through inhibition of SERCA2A Ca^{2+} uptake. The central finding in this study was that Ech A suppressed phosphorylation of PLB at both Ser16 and Thr17, thereby inhibiting SERCA2A activity.

In the ex vivo cardiac experiments, we found the negative inotropic activity of Ech A (Fig. 1). This negative inotropic effect of Ech A is similar to that observed in the clinic when used antihypertensive ACE inhibitors (e.g., ramiprilat and candesartan) are used [41]. As demonstrated here, the short activation time (<5 min) and reversibility of Ech A suggest that the effect of Ech A is direct target effect on rather than an indirect genomic effect. Despite of the negative inotropic effect, Ech A did not induce an arrhythmic phenotype in hearts treated with the compound. In our previous study, Ech A did not induce any cellular toxicity in A7r5 cells (rat aortic vascular smooth muscle cell line) or H9c2 cells (rat cardiomyoblasts) even up to 100 μM dose for 24 h [25]. All of these properties of Ech A suggest a potential clinical use.

The single cardiac myocyte experiments unmasked the negative inotropic effects of Ech A similar to the whole heart experiments (Figs. 2 and 3). These results suggest that Ech A

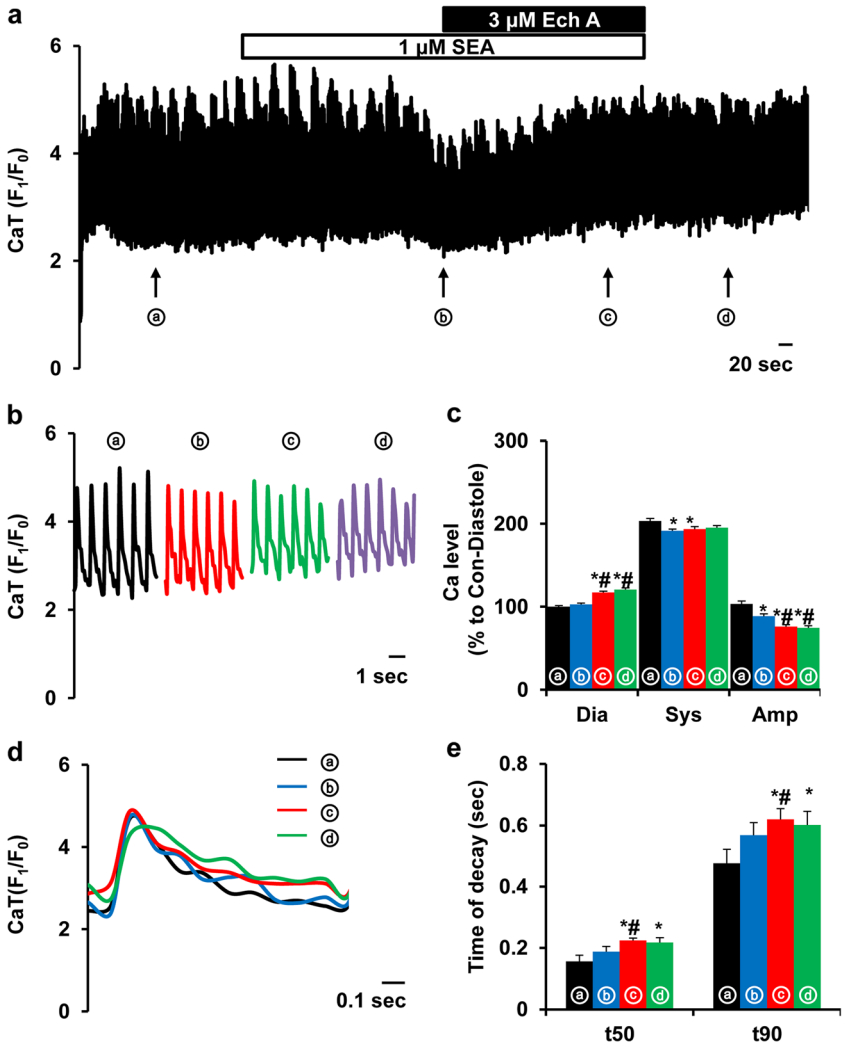

Fig. 5 SEA400 did not affect Ech A-induced diastole [Ca^{2+}] elevation nor delayed time of Ca^{2+} decay. **a** Representative Ca^{2+} traces in stimulated cardiac myocytes during the NT control (ⓐ), SEA400 (1 μM) (ⓑ), SEA400 with Ech A (ⓒ), and washout (ⓓ) periods. **b** Representative CaT traces of the Con, SEA400 (1 μM), SEA400 with Ech A, and washout periods. **c** Diastolic (*Dia*), systolic (*Sys*), and Ca^{2+} amplitude (*Amp*) level comparison. **d** Superimposed CaT single traces of the NT Con (ⓐ), SEA400 (ⓑ), SEA400 with Ech A, (ⓒ), and washout (ⓓ) periods. **e** CaT decay time at 50 and 90 % (*p<0.05 vs. Con, #p<0.05 vs. SEA. n=5)

directly targets LV myocyte function and negatively modulates cell contractility via intracellular Ca^{2+} handling in the absence of sinoatrial rhythmic activity.

LTCC, NCX1, and SERCA2A are known major players, which regulate Ca^{2+} influx and efflux during cardiac E-C coupling. Using patch clamp, we showed that Ech A did not alter Ca^{2+} influx through the LTCC (Fig. 4). Furthermore, the effects of Ech A Ca^{2+} dynamics persisted even in the presence of NCX inhibitors SEA0400 and $NiCl_2$ (Fig. 5 and Supplementary Fig. 5). Moreover, the manner in which $NiCl_2$ and SEA400 disrupted Ca^{2+} handling was markedly different from that observed in myocytes treated with Ech A. These results excluded both NCX and LTCC as a target of Ech A and indicated SERCA2A to be the possible target of Ech A.

SERCA Ca^{2+} uptake experiments (Fig. 6a, b) clearly demonstrated a delay in Ca^{2+} uptake into the SR in the Ech A-treated samples. This evidence supports the hypothesis that SERCA activity is the primary target of Ech A in the myocyte and likely mediates the observed negative inotropic effect of the compound. SERCA2A activity is primarily regulated by phospholamban, a 52 amino acid phosphoprotein, which is closely associated with the SR membrane [39, 15]. Dephosphorylated PLB suppresses SERCA activity, whereas phosphorylation of PLB reverses this inhibition. Two phosphorylation sites of PLB, Ser16 and Thr17, are phosphorylated by PKA and CaMKII, respectively [18]. To understand the molecular basis of SERCA2A inhibition by Ech A, we compared protein level of catalytic subunit of PKA α/β and the phosphorylation status of PLB-Ser16, PLB-Thr17, and CaMKII. The immunoblot results clearly show that Ech A reduced phosphorylation of PLB-Ser16 and PLB-Thr17. The levels of total SERCA2A, catalytic subunit of PKA α/β, and phosphorylated CaMKII were not affected by Ech A (Fig. 6c, d). It is known that increased [Ca^{2+}]i enhance PLB-Thr17 phosphorylation through CaMKII activation [42]. However, our result showed no significant alteration of phospho-

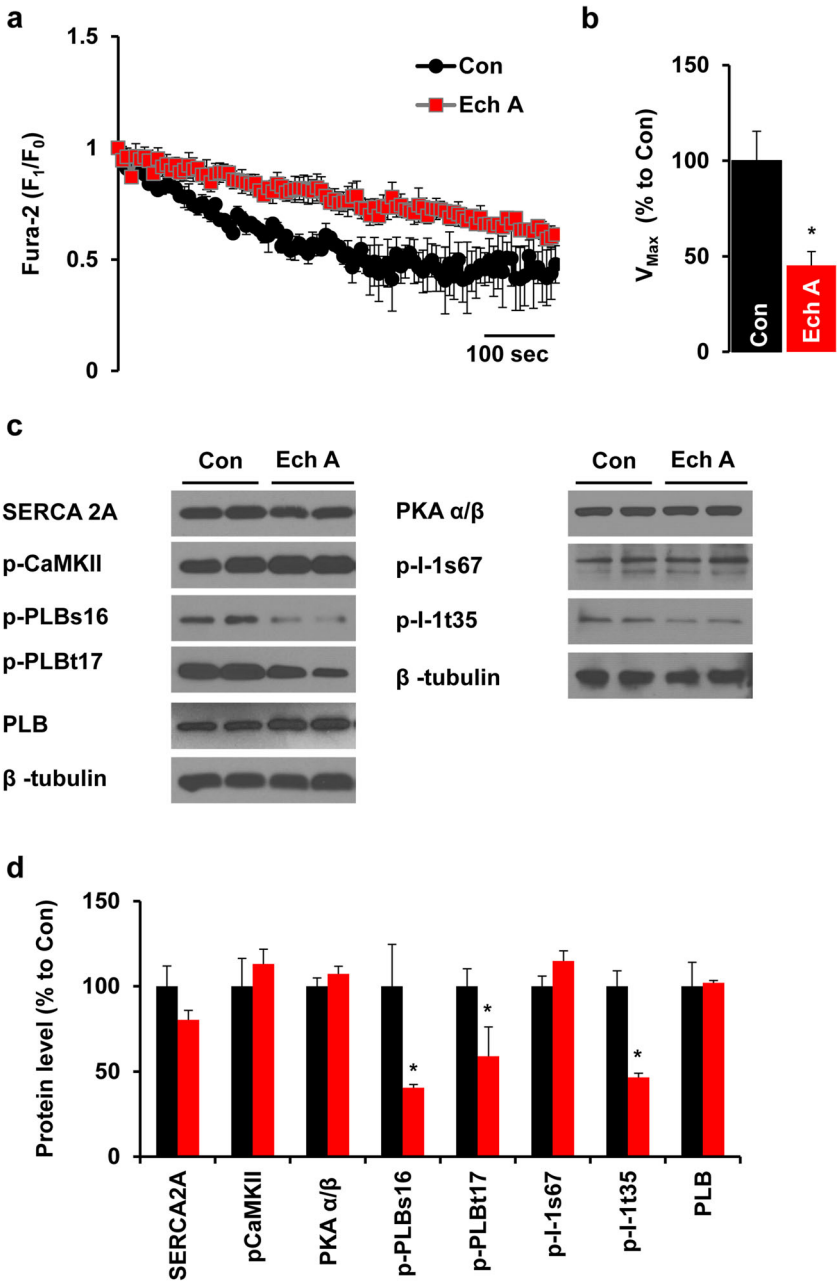

Fig. 6 Ech A inhibited SERCA Ca^{2+} uptake activity through regulating phosphorylation of phospholamban Ser16 and Thr17. **a** Trace of SERCA Ca^{2+} uptake measurement in the presence or absence of Ech A (3 μM). **b** Maximal velocity of Ca^{2+} uptake. **c** Representative immunoblots of protein expression levels of SERCA2A, phosphorylated CaMKII (p-CaMKII), serine 16 or threonine 17 phosphorylated phospholamban (p-PLBs16, p-PLBt17), total phospholamban (PLB), PKA α/β, serine 67 or threonine 35 phosphorylated I-1 (p-I-1s67, p-I-1t35), and β-tubulin from 10 min of NT control- or Ech A- (3 μM) treated rat hearts. **d** Normalized protein level ratio (*$p<0.05$ vs. Con, $n=3$)

CaMKII level or enhanced phosphorylation of PLB in Ech A-treated heart. These result suggested there is no mechanistic relationship between the Ech A-induced rise in resting Ca^{2+} level and the reduced phosphorylation level of PLB in the present results.

To figure out whether decreased phosphorylation of PLB is due to enhancement of dephosphorylation, we further tested status of PP1, the major phosphatase for PLB. The activity of PP1 is regulated by I-1. Phosphorylation of I-1 at Thr35 activates I-1 activity, while phosphorylation at Ser67 inhibits it [33, 7, 32]. Ech A treatment reduced phosphorylation of Thr35 site of I-1 but Ser67 (Fig. 6c, d). These results suggested that Ech A suppressed SERCA2A activity through its enhanced effect on PLB dephosphorylation. However, the more detailed mechanism of this regulation should be studied in further study.

The cardiac SERCA has been indicated to be a primary player in protecting the heart against I/R injury. Therefore, we speculated that the novel effect of Ech A on SERCA2A may also be cardioprotective against I/R injury [21, 31]. Indeed, Ech A pretreatment significantly attenuated myocardial I/R injury as measured by infarct size (Fig. 7). A similar type of cardioprotection associated with SERCA inhibition has been previously reported [12, 13]. In their study, pretreatment with

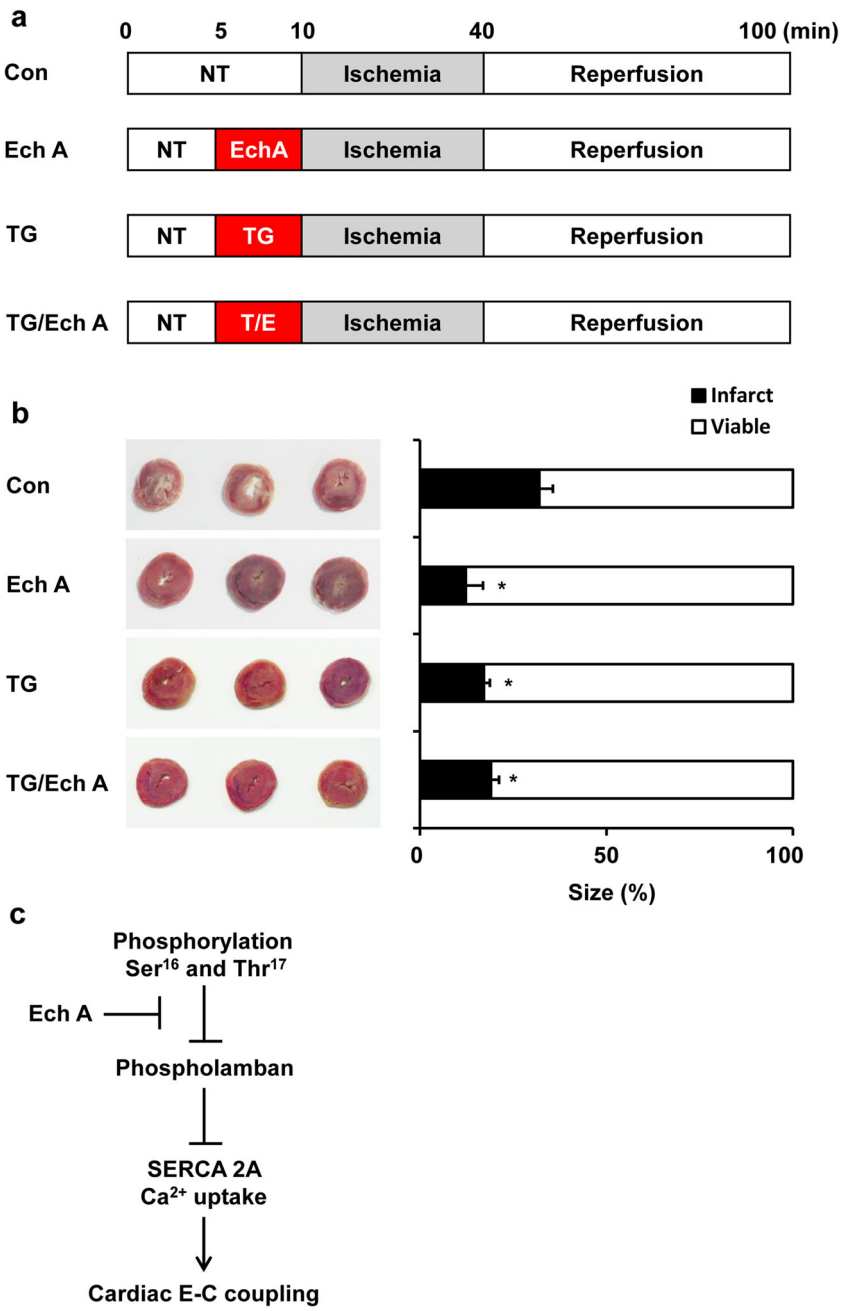

Fig. 7 Ech A attenuates ischemia/reperfusion (I/R) injury. **a** Experimental design for I/R treatment. **b** Images of TTC-stained myocardial tissue slices. *Red*: viable area. *Pale white*: infarcted area. Infarct size comparison in Con-, Ech A- (3 μM), TG- (100 nM), TG with Ech A-treated hearts. **c** Proposed novel Ca^{2+} handling mechanism of Ech A (*$p<0.05$ vs. Con, $n=3$)

SERCA inhibitors, cyclopiazonic acid and TG, successfully decreased I/R injury in rat heart. TG specifically inhibits SERCA activity by stabilizing the E2 conformational state [27]. The effect of Ech A was comparable with the effect of thapsigargin treatment or with co-treatment with TG and Ech A in both its effect on MI size and on CaT dynamics (Fig. 7 and Supplementary Fig. 6). Interestingly, combined treatment with TG and Ech A did not have an additive effect. This may suggest that Ech A and TG share a common target, SERCA 2A.

Because of its intracellular Ca^{2+}-modulating potential, SERCA inhibitors are considered as cancer therapeutic agents [27, 10, 11, 40]. In addition, recent study suggested that SERCA inhibitor has potential to inhibit autophagy [16], which may give clue to use the SERCA inhibitor for regulating maladaptive autophagy in cardiovascular disease [30]. This multiple implication of SERCA might extend the application of Ech A to various clinical fields.

In summary, we discovered a novel mechanism of action for Ech A that regulated phosphorylation of phospholamban Ser16 and Thr17 resulting in a delay in Ca^{2+} uptake by SERCA2A. Consequently, Ech A induces a negative inotropic effect (Fig. 7c).

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Ethical standards All experimental procedures were reviewed and approved by the Institutional Review Board of Animals, Inje University College of Medicine. Procedures were performed according to the guidelines of the Institutional Review Board on the ethical use of animals.

Conflict of interest None declared.

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