



Cardiovascular pharmacology

Alterations of contractions and L-type Ca^{2+} currents by murrayafoline-A in rat ventricular myocytes

Min-Jeong Son^{a,c,1}, Bojjibabu Chidipi^{a,1}, Joon-Chul Kim^a, Tran Thu Huong^b, Bui Huu Tai^a, Young Ho Kim^a, Joung Real Ahn^c, Nguyen Manh Cuong^b, Sun-Hee Woo^{a,*}

^a College of Pharmacy, Chungnam National University, 99 Daehak-ro, Yuseong-gu, Daejeon 305-764, South Korea

^b Department of Bioactive Products, Vietnam Academy of Science and Technology, 18 Hoang Quoc Viet Rd., Hanoi, Vietnam

^c Department of Physics, Sungkyunkwan University, Suwon, South Korea

ARTICLE INFO

Article history:

Received 13 March 2014

Received in revised form

2 July 2014

Accepted 2 July 2014

Available online 10 July 2014

Keywords:

Murrayafoline-A (PubChem CID: 375150)

Positive inotropy

Ventricular myocytes

L-type Ca^{2+} current

ABSTRACT

We examined the effects of murrayafoline-A (1-methoxy-3-methylcarbazole, Mu-A), which is isolated from the dried roots of *Glycosmis stenocarpa*, on cell shortenings and L-type Ca^{2+} currents ($I_{\text{Ca,L}}$) in rat ventricular myocytes. Cell shortenings and $I_{\text{Ca,L}}$ were measured using the video edge detection method and patch-clamp techniques, respectively. Mu-A transiently increased cell shortenings in a concentration-dependent manner with an EC_{50} of $\sim 20 \mu\text{M}$. The maximal effect of Mu-A, approximately 175% of the control, was observed at $\geq 100 \mu\text{M}$. The positive inotropic effect of Mu-A (25 μM) reached a maximum after ~ 2 -min exposures, and then decayed after a ~ 1 -min steady-state. During the Mu-A-induced positive inotropy, the rate of contraction was accelerated, whereas the rate of relaxation was not significantly altered. To understand the possible mechanism for the Mu-A-induced positive inotropy, the $I_{\text{Ca,L}}$ was assessed. Mu-A transiently enhanced the $I_{\text{Ca,L}}$. Concentration-dependence of the increase in $I_{\text{Ca,L}}$ by Mu-A was similar to that of positive inotropic effect of Mu-A. The maximal effect of Mu-A (25 μM) on $I_{\text{Ca,L}}$ was observed at 2–3 min after the application of Mu-A. A partial inhibition of $I_{\text{Ca,L}}$ using verapamil (1 μM) induced a right shift of concentration-response curve of the positive inotropic effect of Mu-A and significantly attenuated the effect. These results suggest that Mu-A may transiently enhance contractility, at least in part, by increasing the Ca^{2+} influx through the L-type Ca^{2+} channels in rat ventricular myocytes.

© 2014 Elsevier B.V. All rights reserved.

1. Introduction

Murrayafoline-A (1-methoxy-3-methylcarbazole, Mu-A) is a known monomeric carbazole alkaloid found in several Rutaceae plants including *Murraya euchrestifolia* Hayata and *Glycosmis stenocarpa* (Furukawa et al., 1985; Cuong et al., 2004). Previous studies have reported that the long-term (15–17 h) treatment of Mu-A has growth inhibitory effects on human fibrosarcoma cells ($\text{IC}_{50} = 118.5 \mu\text{M}$; Cui et al., 2002) and colon cancer cells (Choi et al., 2010). It has also been demonstrated that Mu-A exhibits selective cytotoxicity against MOLT-4 (leukemia) and HOP-18 (lung cancer) cells (log GI_{50} of < -8.60 and -6.54 , respectively) with marginal cytotoxicity against other cancer cell lines (Itoigawa et al., 2000). One of the carbazole alkaloid compounds, murrayaquinone-A, has been shown to alter contractility in guinea-pig

papillary muscle (Takeya et al., 1989). So far, there have been no reports on the effect of Mu-A on cardiac cell function.

The contraction of mammalian cardiac myocytes is controlled by a sequence of events called excitation-contraction coupling (E-C coupling). During an action potential, the depolarization of the cell membrane activates voltage-gated L-type Ca^{2+} channels. Influx of Ca^{2+} through the Ca^{2+} channels triggers Ca^{2+} release from the sarcoplasmic reticulum (SR), resulting in transient increase in intracellular Ca^{2+} concentration and subsequent contraction (Fabiato, 1985; Cannell et al., 1987; Beuckelmann and Wier, 1988; Näbauer et al., 1989; Niggli and Lederer, 1990; Cleemann and Morad, 1991). The cytosolic Ca^{2+} is then removed from the cytosol via the Ca^{2+} ATPase pumps on the SR membrane and the Na^{+} - Ca^{2+} exchangers on the cell membrane, which leads to the relaxation of myocytes (Lakatta, 1996).

The purpose of this study was to examine whether Mu-A alters the contraction of ventricular myocytes and to determine possible mechanism for Mu-A-induced inotropic response. We measured cell shortenings and L-type Ca^{2+} currents ($I_{\text{Ca,L}}$) in isolated rat ventricular myocytes using video edge detection and patch-clamp

* Corresponding author. Tel.: +82 42 821 5924; fax: +82 42 823 6566.

E-mail address: shwoo@cnu.ac.kr (S.-H. Woo).

¹ The authors equally contributed to this study.

techniques, respectively. Our data demonstrate that both cell shortening and $I_{Ca,L}$ are transiently increased by Mu-A in rat ventricular myocytes.

2. Materials and methods

2.1. Cell isolation

Rat ventricular myocytes were enzymatically isolated from male Sprague-Dawley rats (200–300 g) as described previously (Woo et al., 2002). Briefly, rats were deeply anesthetized with sodium pentobarbital (150 mg/kg, i.p.), chest cavities were opened, and hearts were excised. This surgical procedure was carried out in accordance with university ethical guidelines. The excised hearts were retrogradely perfused at 7 ml/min through the aorta (at 36.5 °C), first for 3 min with Ca^{2+} -free Tyrode solution composed of 137 mM NaCl, 5.4 mM KCl, 10 mM HEPES, 1 mM $MgCl_2$, and 10 mM glucose (pH 7.3), and then with Ca^{2+} -free Tyrode solution containing collagenase (1.4 mg/ml, Type I; Roche, Indianapolis, IN) and protease (0.14 mg/ml, Type XIV; Sigma-Aldrich, St. Louis, MO) for 12 min, and finally with Tyrode solution containing 0.2 mM $CaCl_2$ for 8 min. The ventricles of the digested heart were then cut into several sections and subjected to gentle agitation to dissociate the cells.

2.2. Measurement of cell shortenings

Isolated myocytes were continuously superfused with normal Tyrode solution (see above) containing 2 mM Ca^{2+} . Cells were field-stimulated at 1 Hz with two parallel Pt wires connected with an electrical stimulator (Stimulator I, Hugo Sach Elektronik, March-Hugstetten, Germany) at room temperature. Single-cell

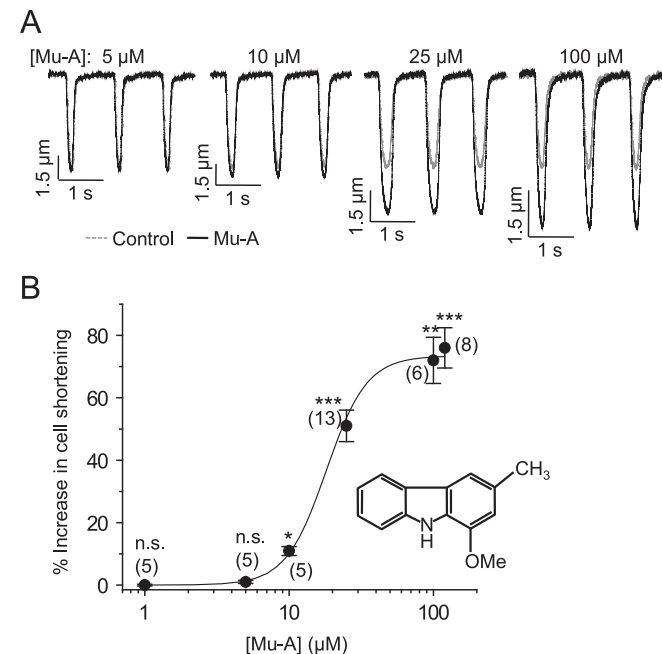


Fig. 1. Concentration-dependent increases in cell shortenings by Mu-A in rat ventricular myocytes. (A) Representative contraction traces recorded immediately before the exposure to Mu-A (Control, gray dashed line) and at the time when a maximal increase in cell shortening by Mu-A is observed (black solid line). The y scale (μm) refers to the magnitude of cell shortening. (B) Concentration-dependent enhancement of cell shortenings (% increase) by the applications of Mu-A (1, 5, 10, 25, 100, and 120 μM). * $P > 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. control. The 'n.s.' indicates 'not significant'. The sigmoidal curve represents the fit of the Hill equation. The number written in the parenthesis indicates number of cells. Inset: structure of Mu-A (1-methoxy-3-methylcarbazole).

shortenings were measured with a video edge detector (Model VED-105, Crescent Electronics, Sandy, UT) connected with a CCD camera (LCL902C, Till Photonics, Graefelfing, Germany) and a video monitor (ViewFinder III, Polychrome V system Till Photonics) as previously reported (Woo and Lee, 1999; Son et al., 2011). The cell shortenings were recorded using a PC software pClamp 9 (Molecular Devices Sunnyvale, CA) connected to an A/D converter (Digidata 1322 A, Molecular Devices). Analyses of the traces were performed by Clampfit 9 (pClamp, Molecular Devices) and Origin software (ver 6.0, Microcal™). EC_{50} values were calculated by fitting the averaged concentration-dependence curve to the Hill equation.

2.3. Measurement of $I_{Ca,L}$

$I_{Ca,L}$ was recorded in the whole-cell configuration of the patch-clamp technique (Hamill et al., 1981) using an EPC7 amplifier (HEKA, Elektronik, Lambrecht/Pfalz, Germany). In some experiments $I_{Ca,L}$ was recorded with a perforated patch-clamp method

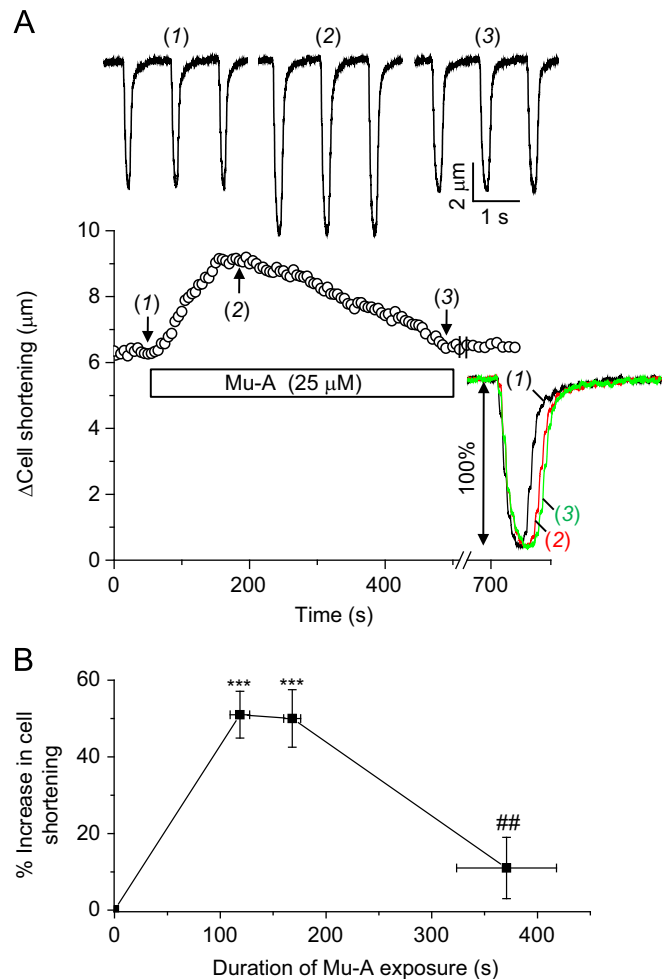


Fig. 2. Time course of Mu-A-induced inotropic effects. (A) Upper panel: representative cell shortenings recorded under control condition (1), and during maximal positive inotropy (2) and maximal negative inotropy (3) in Mu-A (25 μM) as marked by corresponding numbers in the time course (lower panel). Lower panel: time course of the change in the peak values of cell shortenings by Mu-A. Inset: superimposed traces ((1), (2); red) and (3); green) after normalization to each peak value. (B) Mean increases in cell shortenings and averaged time courses of the effects of 25 μM Mu-A. Values represent the time-to-peak effect, the onset of the negative inotropic effect of Mu-A, and the time when the negative inotropy reaches a steady-state. *** $P < 0.001$ vs. control. ## $P < 0.01$ vs. values at maximal positive inotropy. Data are mean \pm S.E.M. ($n = 12$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

(Fig. 4). The patch pipettes were made of glass capillaries (Kimble Glass, Rockwood, TN) to have resistance of 2.5–3 M Ω when filled with the internal solution containing 110 mM CsCl, 20 mM tetraethylammonium (TEA)-Cl, 20 mM HEPES, 5 mM MgATP, and 15 mM ethylene glycol tetraacetic acid (EGTA), and the pH adjusted to 7.2 with CsOH. EGTA was added to the internal solution to buffer micromolar amounts of divalent cations that generally contaminate pipette-filling solutions. We used 15 mM EGTA in order to buffer intracellular Ca²⁺ and prevent indirect change in $I_{Ca,L}$ by alterations in bulk myoplasmic Ca²⁺ concentration (Adachi-Akahane et al., 1996). For the perforated patch-clamp experiments 250 μ g/ml amphotericin-B (Sigma-Aldrich) was added to the internal solutions. Patch pipettes were pulled by using the Flaming/Brown micropipette puller (model P-97, Sutter Instrument Co., Novato, CA). Outward K⁺ currents were suppressed by replacing internal K⁺ with Cs⁺ and TEA⁺, and inward rectifier K⁺ current was suppressed by replacing external K⁺ with Cs⁺. The Na⁺ current was inactivated by holding the membrane potential at –40 mV. Trains of test pulses were 0 mV for 120 ms with 0.1 Hz. The $I_{Ca,L}$ was fully sensitive to 20 μ M nifedipine (data not shown) and to 200 μ M Cd²⁺ (Lee et al., 2008). Measurement of $I_{Ca,L}$ was carried out 5–6 min after rupture of the membrane with the patch pipette, when the rundown of $I_{Ca,L}$ was slowed and stabilized. Generation of the voltage-clamp protocol and acquisition of data were carried out using the pClamp 9 (Molecular Devices) combined with the A/D converter (Digidata 1322A, Molecular Devices). The series resistance was 1.5–3 times the pipette resistance and was electronically compensated through the amplifier. The current signals were digitized at 10 kHz and low-pass filtered at 1 kHz. Data analyses were carried out using the Clampfit software (pClamp 9, Molecular Devices). To measure the inactivation time constant of $I_{Ca,L}$ single exponential curve fitting was performed by Origin program (ver 6.0, Microcal™).

2.4. Isolation of Mu-A

Roots of *G. stenocarpa* were collected and identified as previously described (Cuong et al., 2004). Dried powdered roots (2 kg) of *G. stenocarpa* (Drake) Tan. were extracted with MeOH (4 l; 8 h; room temperature), filtered and evaporated *in vacuo*. The suspension of the methanol residue (185 g) in MeOH/H₂O (1: 1, v/v; 1.2 l) was successively extracted with n-hexane (350 ml), chloroform (300 ml), and butanol (200 ml) to give hexane (40 g), chloroform (35 g), and butanol (38 g) extracts. The hexane extract (20 g) was chromatographed on silica gel (250 g, Merck silica 80–120 mesh)

Table 1
Effects of Mu-A on the kinetics of cell contraction and relaxation.

	Control	Mu-A	
		First positive inotropy	Second negative inotropy
Time-to-peak (ms)	146 ± 12.0	171 ± 13.1 ^a	177 ± 11.3 ^b
Time-to-relaxation (ms)	182 ± 8.76	224 ± 14.9 ^a	258 ± 16.5 ^a
Rate of contraction (μ m/s)	41.7 ± 4.08	50.1 ± 4.50 ^c	35.7 ± 3.03 ^{c,d}
Rate of relaxation (μ m/s)	33.0 ± 2.67	37.8 ± 2.61	24.6 ± 2.29 ^{c,d}

Values are evaluated at the first (positive inotropy) and second (negative inotropy) steady-state of inotropic responses in Mu-A (25 μ M). Data represent mean ± S.E.M. (n = 12).

^a P < 0.01 vs. control (paired t-test).

^b P < 0.0001 vs. control (paired t-test).

^c P < 0.05 vs. control (paired t-test).

^d P < 0.01 vs. "Mu-A, 2 min" (paired t-test).

using a gradient of hexane and EtOAc (100:0–0:100 by volume) as eluent to give 11 fractions. Fraction 3 was further rechromatographed on flash silica gel, eluted with isocratic eluent (mixture of n-hexane/EtOAc, 10/1, v/v), to yield Mu-A (a white crystal-like powder, 3.6 g, 0.36%). Detailed physicochemical and structural information on Mu-A has been described previously (Cuong et al., 2004).

2.5. Solutions and reagents

Stock solutions of Mu-A were made in dimethyl sulfoxide (DMSO). The stock solutions were diluted in the external normal Tyrode solution to make the final testing solutions. The concentrations of DMSO in the testing solutions were < 0.1% (v/v). The same concentrations of DMSO were added to control external solutions. The drug solutions were applied to the cells by superfusion using a custom-made solution switching apparatus. Rapid solution exchange apparatus (Cleemann and Morad, 1991) was

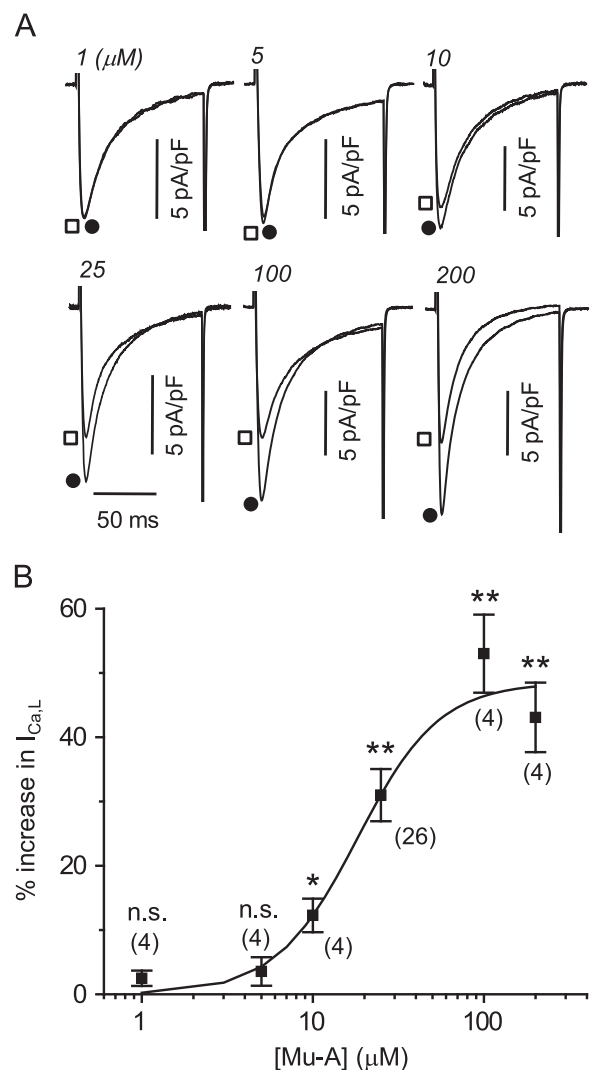


Fig. 3. Concentration-dependence of the stimulatory effect of Mu-A on $I_{Ca,L}$. (A) Representative $I_{Ca,L}$ at 0 mV (holding potential: –40 mV) recorded in each control conditions (open squares) and after the treatments of Mu-A at the concentrations of 1, 5, 10, 25, 100, and 200 μ M (filled circles). The y scale (pA/pF) refers to peak density of $I_{Ca,L}$. (B) Concentration-dependence curve for the Mu-A-induced $I_{Ca,L}$ increase (%). The sigmoidal curve represents the fit of the Hill equation. The numbers in the parentheses indicate the number of cells analyzed. Currents are measured in whole-cell patch-clamped rat ventricular myocytes. *P < 0.05, **P < 0.01 vs. control. 'n.s.' indicates 'not significant'.

used for the experiments using verapamil (Sigma-Aldrich). The experiments were performed at room temperature (22–25 °C).

2.6. Statistics

The numerical results are presented as mean \pm standard error of the mean (S.E.M.) (n =number of cells). A paired or unpaired Student's t -test was used to evaluate the statistical significance of the differences between the means, depending on the properties of the data. Differences at $P < 0.05$ were considered to be statistically significant.

3. Results

3.1. Transient increase in cell shortening by Mu-A

Fig. 1 illustrates the Mu-A concentration–response curve that is obtained after the cell shortenings have reached their maximal positive inotropic response in the presence of given concentrations in rat ventricular myocytes. Mu-A significantly increased the cell shortenings at concentrations of $\geq 10 \mu\text{M}$ (Fig. 1A and B). The resulting maximal change was $\cong 173\%$ of the control, and the maximal effect of the Mu-A was observed at $\geq 100 \mu\text{M}$ (Fig. 1B). The EC_{50} value was $18.1 \pm 1.07 \mu\text{M}$.

The positive inotropic effect of Mu-A was transiently observed at the effective concentrations when the drug solutions were applied for a prolonged period. Fig. 2 shows the time course of the positive inotropic effect by Mu-A. The positive inotropic effect of the Mu-A (25 μM) reached the maximum at ~ 2 min after the onset of the applications (Fig. 2A and B). The maximal effect was maintained for further ~ 1 min, and then the positive inotropic effect decayed. This negative inotropy reached steady-state at ~ 6 min after the onset of the applications (Fig. 2B).

Mu-A significantly enhanced the rate of contraction, but not the rate of relaxation during the positive inotropic phase (Table 1). The time-to-peak of contraction and time-to-relaxation were prolonged during the positive inotropic phase (Table 1). During the negative inotropic phase the rate of contraction and the rate of relaxation were decreased again (Table 1). During this negative inotropic phase, the time-to-peak of the contraction was not

changed further, whereas the time-to-relaxation continued to increase (Table 1).

3.2. Transient increase in $I_{\text{Ca,L}}$ by Mu-A

Ca^{2+} influx through the voltage-gated L-type Ca^{2+} channels during action potential is a major control mechanism of the SR Ca^{2+} release that activates cell shortening. To gain insight into the mechanism underlying the Mu-A-induced positive inotropy, we examined whether Mu-A alters $I_{\text{Ca,L}}$ using the patch-clamp technique in rat ventricular myocytes. Fig. 3A illustrates the representative $I_{\text{Ca,L}}$ traces recorded in the control condition when $I_{\text{Ca,L}}$ become maximal after the applications of 1, 5, 10, 25, 100, and 200 μM Mu-A under whole-cell patch-clamp. The concentration–response curve, which was obtained after $I_{\text{Ca,L}}$ reached its maximal value, demonstrated concentration-dependent enhancement of $I_{\text{Ca,L}}$ in the presence of Mu-A (Fig. 3B). The resulting EC_{50} value was $18.2 \pm 5.04 \mu\text{M}$, which was almost the same as the EC_{50} value for the positive inotropic effect of Mu-A. The maximal increase in $I_{\text{Ca,L}}$ by Mu-A, $\cong 148\%$ (at 0 mV) of the control value, was observed at $\geq 100 \mu\text{M}$ (Fig. 3B).

We further investigated the time course of the effects of Mu-A on $I_{\text{Ca,L}}$ to examine whether the positive inotropic effect of Mu-A correlated with changes in $I_{\text{Ca,L}}$. For the prolonged experiments, we used the perforated patch configuration to reduce the rundown of $I_{\text{Ca,L}}$. Fig. 4 shows the time course of the effect of 25 μM Mu-A on the peak $I_{\text{Ca,L}}$ under the perforated configuration, demonstrating a transient enhancement in $I_{\text{Ca,L}}$ by exposure to Mu-A. Under this condition, 25 μM Mu-A enhanced $I_{\text{Ca,L}}$ to $171 \pm 14.2\%$ of the control values ($n=9$; Fig. 4B). The % increase in $I_{\text{Ca,L}}$ by Mu-A, evaluated in the perforated patch-clamp, was significantly larger than that measured in the whole-cell configuration (see % change at 25 μM in Fig. 3B; $P < 0.01$, unpaired t -test). The time-to-maximal effect of Mu-A on $I_{\text{Ca,L}}$ was observed at 2.5 ± 0.3 min ($n=9$) after the onset of the application. After 4.9 ± 0.7 -min ($n=9$) application of Mu-A, the current was again decreased until the second steady-state (to $126 \pm 16.0\%$ of control value; Fig. 4B). Inactivation time constant of $I_{\text{Ca,L}}$ during the stimulatory (25.4 ± 0.78 ms) and inhibitory (27.3 ± 0.73 ms) phases of the Mu-A effect on $I_{\text{Ca,L}}$ was not different from that under control conditions (25.3 ± 0.84 ms; $n=18$, $P > 0.05$, paired t -test). These results suggest that the Mu-A-induced positive inotropy may be mediated by an increase in $I_{\text{Ca,L}}$.

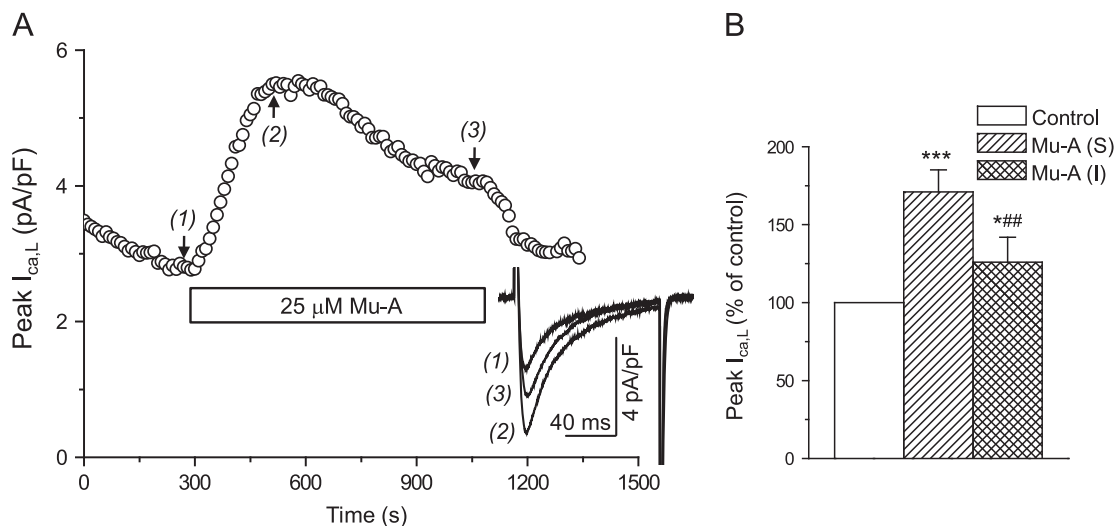


Fig. 4. Time course of the effect of Mu-A on $I_{\text{Ca,L}}$. (A) Time course of the effect of Mu-A (25 μM) on the peak $I_{\text{Ca,L}}$, showing an enhancement of $I_{\text{Ca,L}}$ followed by a decay of the current. Cells are voltage-clamped using perforated mode of patch-clamp technique. Inset: superimposed current traces selected from the points marked by (1), (2), and (3). (B) Mean magnitude of $I_{\text{Ca,L}}$ during stimulatory (S) and inhibitory (I) phases of Mu-A (25 μM) effect on $I_{\text{Ca,L}}$ compared to control $I_{\text{Ca,L}}$ value (100%). * $P < 0.05$, *** $P < 0.01$ vs. Control. ### $P < 0.01$ vs. *Mu-A (P) ($n=9$). Paired t -test.

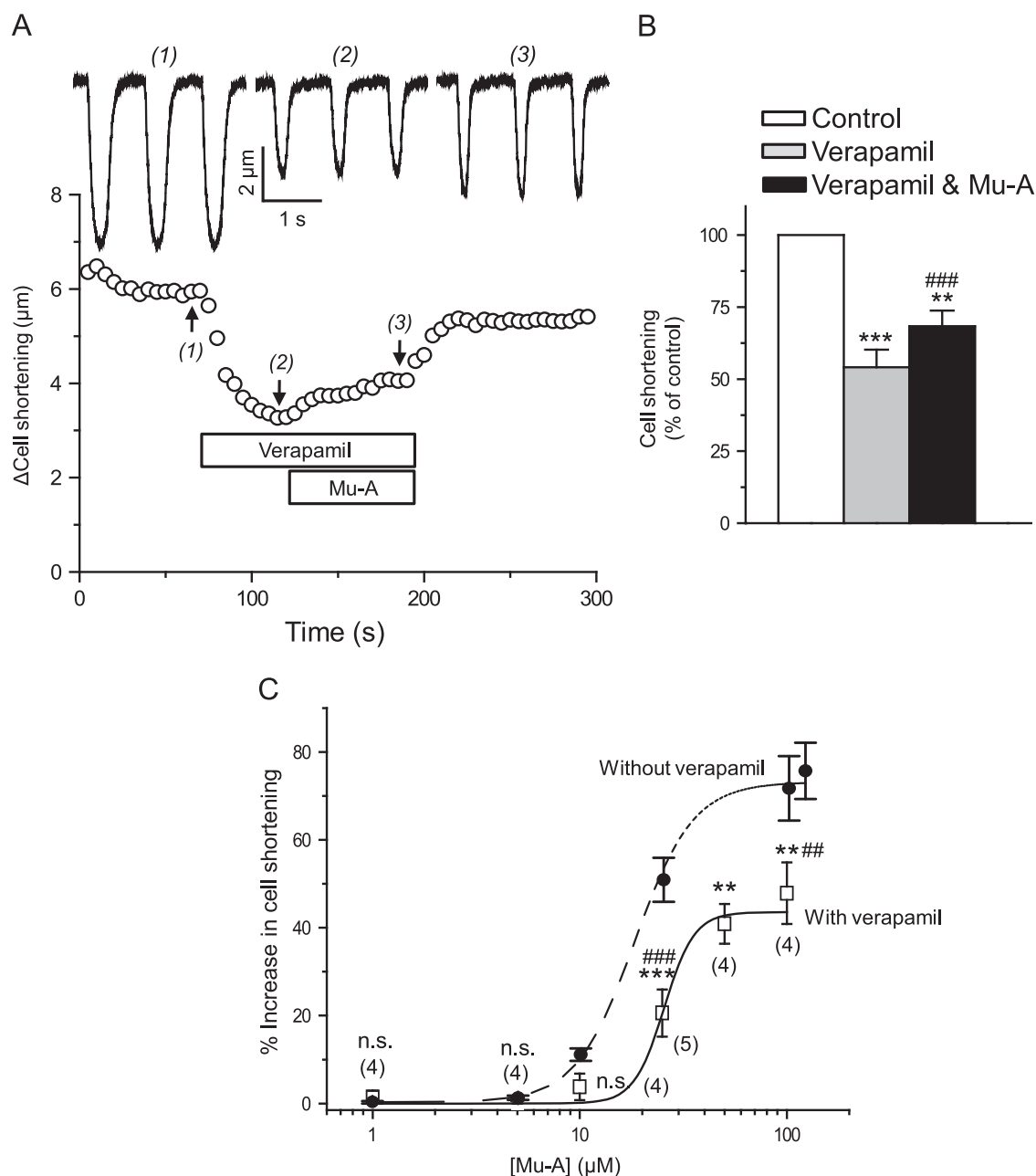


Fig. 5. Reduction of Mu-A-induced positive inotropic effect by the partial inhibition of L-type Ca^{2+} channels. (A) Upper panel: representative traces recorded at the points marked by (1), (2), and (3) in the lower panel. Lower panel: time course of the changes in the cell shortenings by 1 μM verapamil and 1 μM verapamil with 25 μM Mu-A. (B) Mean changes in cell shortenings (% of control) in verapamil (1 μM) and verapamil (1 μM) plus Mu-A (25 μM). ** $P < 0.01$, *** $P < 0.001$, vs. Control. ### $P < 0.001$ vs. "Verapamil" ($n=8$). Paired t -test. (C) Concentration–response curve of positive inotropic effect of Mu-A in verapamil (1 μM)-pretreated myocytes. Dotted curve shows the concentration–response curve measured under control conditions (for detail see Fig. 1B). The sigmoidal curve represents the fit of the Hill equation. The number in the parenthesis indicates number of cells. ** $P < 0.01$, *** $P < 0.001$ vs. control. 'n.s.': not significant.

3.3. Attenuation of Mu-A-induced positive inotropy by partial inhibition of L-type Ca^{2+} channels

To confirm whether the enhancement of I_{CaL} contributes to the positive inotropic effect of Mu-A, the effects of Mu-A on cell shortening were tested after a partial inhibition of the L-type Ca^{2+} channels. We applied submaximal concentrations of verapamil, a Ca^{2+} channel blocker, using a microbarreled rapid solution exchange system (Cleemann and Morad, 1991) to prevent the possible depletion of the SR Ca^{2+} due to treatment of Ca^{2+} channel blocker. At a concentration of 1 μM , verapamil decreased cell shortening by $\cong 46\%$ (at 1 Hz) within 50 s of application (Fig. 5A and B). Additional treatment with 25 μM Mu-A in the presence of 1 μM verapamil

increased the cell shortenings by approximately 20%, compared with the contractility in the presence of verapamil only (Fig. 5A and B). Concentration–response curve of Mu-A-induced positive inotropy was measured in verapamil-pretreated myocytes, showing higher EC_{50} ($25.5 \pm 2.39 \mu\text{M}$) with a right shift of the curve (Fig. 5C). The effects of Mu-A in the presence of verapamil (maximum = $43.5 \pm 4.13\%$ in the fit of the Hill equation) were significantly smaller than those measured in the absence of verapamil (Fig. 5C). When the L-type Ca^{2+} channels were partially blocked by another inhibitor nifedipine, Mu-A-induced positive inotropic effect was also significantly reduced (data not shown).

In verapamil-pretreated cells, the time-to-peak of contraction and the time-to-relaxation were no longer increased by Mu-A

Table 2
Effects of Mu-A on the kinetics of cell contraction and relaxation in the presence of verapamil.

	Control	Verapamil	Verapamil+Mu-A
Time-to-peak (ms)	131 ± 2.78	114 ± 7.41 ^a	111 ± 6.39 ^a
Time-to-relaxation (ms)	226 ± 22.1	185 ± 23.1 ^a	185 ± 22.6 ^b
Rate of contraction (μm/s)	51.1 ± 3.77	38.9 ± 4.10 ^a	50.0 ± 4.03 ^c
Rate of relaxation (μm/s)	30.7 ± 3.88	24.3 ± 2.04	30.6 ± 2.66 ^d

Values are obtained when the negative inotropic effect of verapamil (1 μM) reaches steady-state ("Verapamil") and after 25 μM. Mu-A shows maximal positive inotropic effect in the presence of verapamil ("Verapamil+Mu-A"). Data represent mean ± S.E.M. (n=8).

^a $P < 0.05$ vs. control (paired t -test).

^b $P < 0.01$ vs. control (paired t -test).

^c $P < 0.01$ vs. "verapamil" (paired t -test).

^d $P < 0.05$ vs. "verapamil" (paired t -test).

exposure during positive inotropic phase, which was in contrast with the prolongation of these parameters by Mu-A under control conditions (Table 1). In the presence of verapamil, the rate of contraction and the rate of relaxation were somewhat accelerated by Mu-A (Table 2). Verapamil alone significantly decreased the time-to-peak contraction, time-to-relaxation, and the rate of contraction (Table 2). These results indicate that Mu-A-induced positive inotropic effect is significantly reduced by the partial inhibition of L-type Ca^{2+} channels, suggesting that the enhancement of $I_{\text{Ca,L}}$ may in part contribute to the positive inotropic effect of Mu-A.

4. Discussion

This is the first report to demonstrate that Mu-A elicits increases in cell shortenings and $I_{\text{Ca,L}}$ in cardiac myocytes. The positive inotropy induced by Mu-A was accompanied by an acceleration of the rate of contraction, but not of the rate of relaxation. By prolonged exposure to Mu-A, these stimulatory effects on contractility and $I_{\text{Ca,L}}$ were significantly reduced. Maximal stimulatory effects of Mu-A on both $I_{\text{Ca,L}}$ and cell shortenings were observed after about 2- to 3-min exposures. The EC_{50} values for the contractile response and for the changes of $I_{\text{Ca,L}}$ in Mu-A were also similar. The Mu-A-induced positive inotropic effect was significantly reduced when the L-type Ca^{2+} channels were partially inhibited using submaximal concentrations of verapamil. We conclude that the increase in the Ca^{2+} influx through the L-type Ca^{2+} channel may, at least in part, contribute to the transient enhancement in contractility of ventricular myocytes by Mu-A.

It is reasonable to suppose that the increased influx of Ca^{2+} through the L-type Ca^{2+} channels may enhance the cell contractility by increasing SR Ca^{2+} releases via the Ca^{2+} -induced Ca^{2+} release mechanism during depolarization. However, the possibility of alterations in the Ca^{2+} release process by Mu-A remains to be tested. The release of SR Ca^{2+} is thought to be responsible for about 60–70% of the inactivation of $I_{\text{Ca,L}}$ (Adachi-Akahane et al., 1996). Therefore, the change in the SR Ca^{2+} release is believed to cause a parallel alteration of the inactivation kinetics of $I_{\text{Ca,L}}$ (Sham et al., 1995; Adachi-Akahane et al., 1996). We have not found any significant change in the inactivation time constant of $I_{\text{Ca,L}}$ by Mu-A during the stimulatory and inhibitory phases. This may be partly due to the use of high concentrations of internal EGTA (15 mM) that limits Ca^{2+} diffusion and the Ca^{2+} -dependent inactivation of Ca^{2+} channels.

The decrease in $I_{\text{Ca,L}}$ during the prolonged exposure to Mu-A is also consistent with the gradual decay of the positive inotropic effect induced by the long-term exposure to Mu-A. However, the stimulatory effect of Mu-A on $I_{\text{Ca,L}}$ was maintained for a longer

duration than the positive inotropy. This may suggest that the change in $I_{\text{Ca,L}}$ may not fully explain the inotropic effects of Mu-A. Interestingly, another carbazole alkaloid compound isolated from the Rutaceae plant, murrayaquinone-A, showed triphasic inotropic effects in guinea-pig papillary muscle: first positive, second negative, and third positive phases (Takeya et al., 1989). In this tissue preparation, the maximum positive inotropic effect was developed ~30 min after the murrayaquinone-A (30 μM) treatment, and the second decaying phase was observed until ~90 min (Takeya et al., 1989). The contractility measured at the second phase of the murrayaquinone-A-induced inotropic effects was not smaller than the control contractility, consistent with the effect of Mu-A on the cell contractility (Fig. 2B). However, application of Mu-A did not produce the third positive phase that murrayaquinone-A showed, and it induced much more rapid changes in the contractility compared with murrayaquinone-A, even when taking into account the different experimental preparations and animal species used. In addition, murrayaquinone-A increased the speed of contraction and relaxation during the positive inotropy (Takeya et al., 1989), while Mu-A accelerated only the rate of contraction (Table 1). Our finding on the enhancement in $I_{\text{Ca,L}}$ by Mu-A is consistent with the previous report on the increase of action potential duration by murrayaquinone-A (Takeya et al., 1989). Mu-A did not alter the rate of relaxation during the positive inotropic phase, which may eliminate the possibility that β -adrenergic receptor/protein kinase A (PKA) signaling has a role in the positive inotropic effect of Mu-A. In fact, the pretreatment of β -adrenergic antagonist propranolol did not prevent the positive inotropy in Mu-A (data not shown). In addition, pre-treatment of PKA inhibitor KT-5720 did not alter Mu-A-induced $I_{\text{Ca,L}}$ enhancement (data not shown). Somewhat consistently, the previous report has also shown that the positive inotropy caused by murrayaquinone-A is resistant to metoprolol, a β_1 -adrenergic antagonist (Takeya et al., 1989).

The stimulatory effect of Mu-A on $I_{\text{Ca,L}}$ was found to be greater in the perforated configuration of the patch-clamp technique than in whole-cell mode of the patch-clamp (Figs. 3 and 4). It is known that perforated mode of patch-clamp technique can preserve intracellular context better than whole-cell mode (Lauer et al., 1992). Further investigation is needed to discover the intracellular signaling molecule and cellular mechanisms involved in the stimulatory effects of Mu-A on $I_{\text{Ca,L}}$ and contraction.

Acknowledgments

This work was supported by the National Research Foundation of Korea (NRF) grants funded by the Korea Government (MEST) (2011-0015637 and 2009-0093815), and partly by the grants from Ministry of Science and Technology of Vietnam (52/2011/HD-NDT) and from Institute of Drug Research and Development, Chungnam National University (in 2013).

References

- Adachi-Akahane, S., Cleemann, L., Morad, M., 1996. Cross-signaling between L-type Ca^{2+} channels and ryanodine receptors in rat ventricular myocytes. *J. Gen. Physiol.* 108, 435–454.
- Beuckelmann, D.J., Wier, W.G., 1988. Mechanism of release of calcium from sarcoplasmic reticulum of guinea-pig cardiac cells. *J. Physiol. (Lond.)* 405, 233–255.
- Cannell, M.B., Berlin, J.R., Lederer, W.J., 1987. Effect of membrane potential changes on the calcium transient in single rat cardiac muscle cells. *Science* 238, 1419–1423.
- Choi, H., Gwak, J., Cho, M., Ryu, M.J., Lee, J.H., Kim, S.K., Kim, Y.H., Lee, G.W., Yun, M. Y., Cuong, N.M., Shin, J.G., Song, G.Y., Oh, S., 2010. Murrayaquinone A attenuates the Wnt/ β -catenin pathway by promoting the degradation of intracellular β -catenin proteins. *Biochem. Biophys. Res. Commun.* 391, 915–920.

- Cleemann, L., Morad, M., 1991. Role of Ca^{2+} channel in cardiac excitation-contraction coupling in the rat: evidence from Ca^{2+} transients and contraction. *J. Physiol. (Lond.)* 432, 283–312.
- Cui, C.B., Yan, S.Y., Cai, B., Yao, X.S., 2002. Carbazole alkaloids as new cell cycle inhibitor and apoptosis inducers from *Clausena dunniana* Levl. *J. Asian Nat. Prod. Res.* 4, 233–241.
- Cuong, N.M., Hung, T.Q., Sung, T.V., Taylor, W.C., 2004. A new dimeric carbazole alkaloid from *Glycosmis stenocarpa* roots. *Chem. Pharm. Bull.* 52, 1175–1178.
- Fabiato, A., 1985. Simulated calcium current can both cause calcium loading in and trigger calcium release from the sarcoplasmic reticulum of a skinned canine cardiac Purkinje cell. *J. Gen. Physiol.* 85, 291–320.
- Furukawa, H., Wu, T.S., Ohta, T., Kuoh, C.S., 1985. Chemical constituents of *Murraya euchrestifolia* HAYATA. *Chem. Pharm. Bull.* 33, 4132–4138.
- Hamill, O.P., Marty, A., Neher, E., Sakmann, B., Sigworth, F.J., 1981. Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflüg. Arch.* 319, 85–100.
- Itoigawa, M., Kashiwada, Y., Ito, C., Furukawa, H., Tachibana, Y., Bastow, K.F., Lee, K. H., 2000. Antitumor agents, 203, carbazole alkaloid murrayaquinone A and related synthetic carbazole quinones as cytotoxic agents. *J. Nat. Prod.* 63, 893–897.
- Lakatta, E.G., 1996. Regulation of cardiac relaxation. In: Morad, M., Ebashi, S., Trautwein, W., Kurachi, Y. (Eds.), *Molecular Physiology and Pharmacology of Cardiac Ion Channels and Transporters*. Kluwer Academic Publishers, The Netherlands, pp. 481–511.
- Lauer, M.R., Gunn, M.D., Clusin, W.T., 1992. Endothelin activates voltage-dependent Ca^{2+} current by a G protein-dependent mechanism in rabbit cardiac myocytes. *J. Physiol. (Lond.)* 448, 729–747.
- Lee, S., Kim, J.C., Li, Y., Son, M.J., Woo, S.H., 2008. Fluid pressure modulates L-type Ca^{2+} channel via enhancement of Ca^{2+} -induced Ca^{2+} release in rat ventricular myocytes. *Am. J. Physiol. Cell Physiol.* 294, C966–C976.
- Näbauer, M., Callewaert, G., Cleemann, L., Morad, M., 1989. Regulation of calcium release is gated by calcium current, not gating charge, in cardiac myocytes. *Science* 244, 800–803.
- Niggli, E., Lederer, W.J., 1990. Voltage-independent calcium release in heart muscle. *Science* 250, 565–568.
- Sham, J.S., Cleemann, L., Morad, M., 1995. Functional coupling of Ca^{2+} channels and ryanodine receptors in cardiac myocytes. *Proc. Natl. Acad. Sci. U. S. A.* 92, 121–125.
- Son, M.J., Kim, H.K., Huong, D.T.T., Kim, Y.H., Sung, T.V., Cuong, N.M., Woo, S.H., 2011. Chrysosplenol C increases contraction in rat ventricular myocytes. *J. Cardiovasc. Pharmacol.* 57, 259–262.
- Takeya, K., Itoigawa, M., Furukawa, H., 1989. Triphasic inotropic response of guinea-pig papillary muscle to murrayaquinone-A isolated from *Rutaceae*. *Eur. J. Pharmacol.* 169, 137–145.
- Woo, S.H., Lee, C., 1999. Effects of endothelin-1 on Ca^{2+} signaling in guinea-pig ventricular myocytes: role of protein kinase C. *J. Mol. Cell. Cardiol.* 31, 631–643.
- Woo, S.H., Cleemann, L., Morad, M., 2002. Ca^{2+} current-gated focal and local Ca^{2+} release in rat atrial myocytes: evidence from rapid 2-D confocal imaging. *J. Physiol. (Lond.)* 543, 439–453.