J Pharmacol Sci **109**, 193 – 202 (2009)

Journal of Pharmacological Sciences

Full Paper

Wortmannin Inhibits the Increase in Myofilament Ca^{2+} Sensitivity Induced by Cross-Talk of Endothelin-1 With Norepinephrine in Canine Ventricular Myocardium

Li Chu[1,2], Ikuo Norota[1], Kuniaki Ishii[1], and Masao Endoh[1,*]

[1]*Department of Cardiovascular Pharmacology, Yamagata University School of Medicine, Yamagata 990-9585, Japan*
[2]*Department of Pharmacology, Hebei Medical University, Shijiazhuang 050051, P.R. China*

Received August 30, 2008; Accepted December 3, 2008

Abstract. Endothelin-1 (ET-1) modulates cardiac contractility by cross-talk with norepinephrine (NE) in canine ventricular myocardium. The present experiments were performed to investigate the influence of wortmannin that has inhibitory action on phosphatidylinositol 3-kinase (PI3-K) (IC_{50} = 3 nM) and myosin light chain kinase (MLCK) (IC_{50} = 200 nM) on Ca^{2+} signaling and the inotropic effects of ET-1 induced by cross-talk with NE. Experiments were carried out in isolated canine ventricular trabeculae and indo-1/AM–loaded single ventricular cardiomyocytes. ET-1 alone elicited a transient small negative inotropic effect (NIE). In the presence of NE at low (1 – 10 nM) and high (100 nM) concentrations, ET-1 induced a long-lasting positive inotropic effect (PIE) or a marked sustained NIE, respectively. Wortmannin up to 300 nM did not affect the contractility; and at 1 μM and higher, it decreased the basal contraction without suppressing Ca^{2+} transients. Wortmannin (1 μM) inhibited the long-lasting PIE of ET-1 without affecting the ET-1–induced increase in Ca^{2+} transients. Wortmannin at the same concentration did not affect the ET-1–induced transient and sustained NIE and the PIE mediated by β-adrenoceptor stimulation. These results imply that wortmannin exerts selective inhibitory action on the increase in myofilament Ca^{2+} sensitivity induced by cross-talk of ET-1 with NE probably through an inhibition of MLCK in canine ventricular myocardium.

Keywords: inotropic effect, Ca^{2+} transient, endothelin-1, norepinephrine, wortmannin

Introduction

In the canine ventricular myocardium, endothelin-1 (ET-1) alone elicits only a small transient negative inotropic effect (NIE) (1, 2), but it induces a prominent positive inotropic effect (PIE) or sustained NIE by cross-talk with norepinephrine (NE) (3 – 5). In the presence of NE at threshold or sub-threshold concentrations (1 – 10 nM), ET-1 elicits a long-lasting PIE that is associated with a moderate increase in Ca^{2+} transients and an increase in myofilament Ca^{2+} sensitivity via activation of ET_A receptors (3 – 5). When the concentration of NE is elevated, the inotropic effect of ET-1 is gradually reversed to the NIE, and in the presence of NE at higher concentrations (100 – 1,000 nM), ET-1 induces a pronounced sustained NIE likewise via activation of ET_A receptors (2 – 5).

Since the plasma levels of both ET-1 and NE are elevated in cardiovascular disorders, including congestive heart failure and ischemic heart disease, it is highly likely that ET-1 regulates cardiac contractility by cross-talk with NE under pathophysiological conditions.

The mechanism of the increase of myofilament Ca^{2+} sensitivity has been studied extensively in mammalian ventricular myocardium including that of the rabbit, in which ET-1 alone elicits a marked PIE (6 – 9). Based on these studies, it is postulated that the increase in Ca^{2+} sensitivity induced by ET-1 is due to an intracellular alkalinization and/or an increase in phosphorylation of myofilaments (10, 11), but the signaling process responsible for the Ca^{2+} sensitization has not been fully clarified.

*Corresponding author. mendou@med.id.yamagata-u.ac.jp
Published online in J-STAGE
doi: 10.1254/jphs.08228FP

In smooth muscle cells, it has been shown that myosin light chain (MLC) kinase (MLCK) and Rho kinase act in concert to increase phosphorylation of MLC, which leads to an increase in myofilament Ca^{2+} sensitivity (12). While the role of MLC in cardiac contractile regulation has been studied relatively less, it was shown that MLCK induces phosphorylation of MLC-2 and increases myofilament sensitivity to Ca^{2+} in cardiac skinned fibers (13, 14). Furthermore, the PIE of cardiac α_1-adrenoceptor stimulation that induces an increase in Ca^{2+} sensitivity in cardiac muscle is associated with the MLC-2 phosphorylation (15, 16). ET-1 increases likewise MLC-2 phosphorylation in rat cardiac muscle (10).

Wortmannin is a potent inhibitor of MLCK with an IC_{50} 200 nM in vitro (17); at $1-3\ \mu$M, it inhibits effectively the MLCK activity in intact smooth muscle cells (18, 19), and at 1 μM, it suppressed the increase in myofilament Ca^{2+} sensitivity induced by ET-1 alone in the rabbit ventricular muscle (20). In the present study, we investigated the influence of wortmannin on the inotropic effects of ET-1 induced by cross-talk with NE in the canine ventricular myocardium.

Materials and Methods

This study was conducted in accordance with Guiding Principles for the Care and Use of Laboratory Animals approved by The Japanese Pharmacological Society and the Guidance for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication no. 85-23, revised 1996). The approval for the animal experiments was obtained from the Committee for Animal Experimentation, Yamagata University School of Medicine prior to the experiments.

Isolation of canine cardiac myocytes

Ventricular cardiomyocytes were obtained from mongrel dogs of either sex weighing 7 – 10 kg by previously described procedures (21). Briefly, a portion of the left ventricular free wall that is supplied with the branch of left anterior descending artery was excised, and the artery was cannulated and perfused for approximately 2 min at 37°C by means of a Langendorff apparatus with Tyrode's solution to wash out the blood. The tissue was then perfused with nominally Ca^{2+}-free Tyrode's solution for 8 min at a rate of about 40 ml/min. Tyrode's solution contained 136.5 mM NaCl, 5.4 mM KCl, 0.53 mM $MgCl_2$, 1.8 mM $CaCl_2$, 0.33 mM NaH_2PO_4, 5.0 mM glucose, and 5.0 mM HEPES (pH 7.4); it was bubbled continuously with 100% O_2. The perfusion solution was changed to nominally Ca^{2+}-free Tyrode's solution that contained 1.0 mg/ml collagenase and 0.1 mg/ml protease, and the perfusion was continued for 15 – 20 min at a perfusion rate of 20 ml/min by use of a recirculating system. Finally the muscle piece was perfused with Tyrode's solution that contained 0.2 mM $CaCl_2$ and then cut into small pieces with a scalpel. The cells were rinsed several times with a gradual increase in the Ca^{2+} concentration up to 1.8 mM. The myocytes displaying rod shaped, well-defined striations and no spontaneous contractions were used for the experiments.

Loading of myocytes with indo-1/AM

Myocytes were loaded with indo-1/AM by incubating them in 5 μM indo-1/AM solution for about 3 min at room temperature (25°C). After loading, they were centrifuged at $5 \times g$ for 1 min. The pellet was resuspended in HEPES-Tyrode solution. The myocytes were then laid in the chamber superfused with bicarbonate buffer for about 10 min. The bicarbonate buffer contained 116.4 mM NaCl, 5.4 mM KCl, 0.8 mM $MgSO_4$, 1.8 mM $CaCl_2$, 1.0 mM NaH_2PO_4, 5.0 mM glucose, and 23.8 mM $NaHCO_3$ (pH 7.4) and had been bubbled with 95% O_2 and 5% CO_2.

Simultaneous measurements of cell shortening and Ca^{2+} transients

The myocytes were laid in a perfusion chamber placed on the stage of an inverted microscope (Diaphot TMD 300; Nikon, Tokyo). After 10 min when the cells settled down to attach loosely to the bottom of chamber, the perfusion was started with bicarbonate buffer containing 1.8 mM $CaCl_2$ at a rate of 1 ml/min at room temperature (25°C) and the cells were stimulated electrically by square-wave pulses with voltage about 30% – 40% above the threshold at a frequency of 0.5 Hz. Myocytes that showed the following characteristics were used for the experiments: single rod-shaped cells that were unattached to either adjacent cells or debris and contracted regularly with each electrical stimulus, being quiescent between stimuli.

Fluorescence of indo-1 was excited with the light from a xenon lamp (150 W) at a wavelength of 355 nm, reflected by a 380-nm long-pass dichroic mirror, and detected by a fluorescence spectrophotometer (CAM-230; Japan Spectroscopic Co., Tokyo). Excitation light was applied to myocytes intermittently through a neutral density filter to minimize the photobleaching of indo-1. The emitted fluorescence was collected by an objective lens (CF Fluor DL40, Nikon) and then separated by a 580-nm long-pass dichroic mirror to permit simultaneous measurements of light at both 405-nm and 500-nm wavelengths through band-pass filters.

The emission field was restricted to a single cell with the aid of an adjustable window. The fluorescence ratio

(405/500 nm) was used as an indicator of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) (22). Cells were simultaneously illuminated with red light (wavelength above 620 nm) through the normal bright-field illumination optics of the microscope, and the bright-field images of a myocyte were collected by objective lens and first separated by a 580-nm long-pass dichroic mirror (Omega Optical, Brattleboro, VT, USA). A bright-field cell image was projected onto a photodiode array of the edge detector (C6294-01; Hamamatsu Photonic K.K., Hamamatsu) with 5-ms temporal resolution and the cell length was monitored simultaneously with indo-1 fluorescence.

Data recordings and analysis

Cell length and fluorescence of indo-1 were stored and displayed by means of a computer (Power Macintosh 8100/100AV; Apple Computer Inc., Cupertino, CA, USA) equipped with an A/D converter (MP-100A; BIOPAC Systems, Inc., Santa Barbara, CA, USA) at 200 Hz and analyzed after low-pass filtering (cutoff frequency of 20 Hz). The data used for statistical analysis were obtained by signal-averaging of five successive tracings of cell shortening and Ca^{2+} transients. In the analysis of data, the diastolic cell length and indo-1 fluorescence ratio prior to first application of the agent examined in individual experiments were regarded as baseline values in each myocyte, which are assigned to 100%, and all the data are expressed as a percentage of the baseline values.

Experimental protocols

After an equilibration period of 40 min, myocytes were perfused with the solution containing the agent examined. When the response of myocytes to the agent applied reached a stable level, the indo-1 fluorescence was measured and then the perfusion was switched to the solution that contained an additional agent. The cell length was continuously measured throughout the experiments, while the fluorescence of indo-1 was monitored intermittently to reduce the quenching. The cell length and Ca^{2+} transients were simultaneously recorded at the baseline state and in the presence of the agent when the response had reached a steady level. An increase or decrease in cell shortening is considered to reflect qualitatively the PIE or NIE, which is often referred to as PIE or NIE interchangeably without explanation. Prazosin (300 nM) and wortmannin were allowed to act for 20 min before the application of NE or ET-1 and were present throughout the experiments.

Measurements of inotropic effects in canine ventricular trabeculae

Mongrel dogs (7 – 10 kg) of either sex were used in these experiments. Two to four ventricular trabeculae were excised from the right ventricle of a dog that had been anesthetized with pentobarbital sodium (30 mg/kg, i.v.) and they were mounted in 20-ml organ baths that contained Krebs-Henseleit solution (with 0.057 mM ascorbic acid and 0.027 mM EDTA, disodium salt, to prevent autoxidation of the compounds examined) bubbled with 95% O_2 and 5% CO_2 at 37°C (pH 7.4). Muscle preparations were electrically stimulated with a pulse of 5-ms duration and a voltage 20% above the threshold (approximately 0.4 V) at a frequency of 0.5 Hz. Force of isometric contractions was detected with strain gauge transducers (Shinkoh UL 10 GR; Minebea, Tokyo) and recorded on a thermal pen-writing oscillograph (Recti-Horiz-8K; NEC San-ei Instruments, Tokyo). Muscles were stretched initially under a tension of 5 mN, and the muscle length was adjusted to give 90% of the maximal contractile force during the equilibration period of 60 min. Preparations of canine ventricular trabeculae had the average dimensions of 14.15 ± 2.01 mm in length and 0.74 ± 0.25 mm^2 in cross-sectional area (n = 47, from 13 dogs). In all experiments, the α-adrenoceptor antagonist prazosin (300 nM) was allowed to act for 30 min before the addition of NE, and it was present in the organ bath throughout the experiments. At the beginning of each experiment, the β-adrenoceptor agonist isoproterenol at 100 nM was applied two or three times until reproducible responses were obtained. This procedure was important to confirm the stability of individual preparations.

First, the influence of wortmannin on the PIE induced by cross-talk of ET-1 with NE was investigated. The PIE of ET-1 in the presence of NE at a subthreshold concentration of 1 nM was determined in the absence and presence of wortmannin. Wortmannin was administered 30 min before the addition of NE and was present in the organ bath throughout the experiments.

The concentration–response curve (CRC) for NE mediated by β-adrenoceptors was determined in the absence and presence of wortmannin at 3 μM. After the CRC for NE was completed, NE was washed out for 2 h, and then the maximal response to ISO (ISO_{max}) was obtained by a cumulative administration. The NE-induced inotropic response was calculated as a percentage of the ISO_{max}.

The NIE of ET-1 in the presence of NE at 100 nM was determined in the absence or presence of wortmannin. The first response to NE was determined for 60 min, NE was washed out for 60 min, and it was added again and allowed to act for 15 min before the addition of

ET-1. ET-1 was administered at a single concentration to each preparation. Wortmannin was administered 30 min before the addition of NE and was present in the organ bath throughout the experiments. The inotropic responses to NE and ET-1 were expressed as the percentage of the basal force of contraction before the addition of NE in each preparation.

Drugs

The drugs used were ET-1 (Peptide Institute, Osaka), prazosin hydrochloride (Pfizer Taito, Tokyo); norepinephrine hydrochloride (Nacalai Tesque, Kyoto); wortmannin, (–)-isoproterenol, and protease type XIV (Sigma, Chemical Co., St. Louis, MO, USA); pentobarbital sodium (Tokyo Kasei, Tokyo); indo-1/AM (Dojindo Chemical, Kumamoto); collagenase type II (Worthington Biochemical, Freehold, NJ, USA); dimethyl sulfoxide (DMSO; Wako Pure Chemicals, Osaka). Other reagents used were of the highest grade in purity available commercially.

Statistics

Experimental values are presented as means ± S.E.M. Significant differences between mean values were estimated by a repeated-measures analysis of variance and/or by Student's *t*-test with analytic software STATVIEW J-4.5 (Abacus Concepts, Berkeley, CA, USA). $P<0.05$ was judged to indicate a statistically significant difference between two means.

Results

Influence of wortmannin on baseline contractility and intracellular Ca^{2+} transients

We first investigated the inotropic effects of wortmannin at different concentrations on the baseline

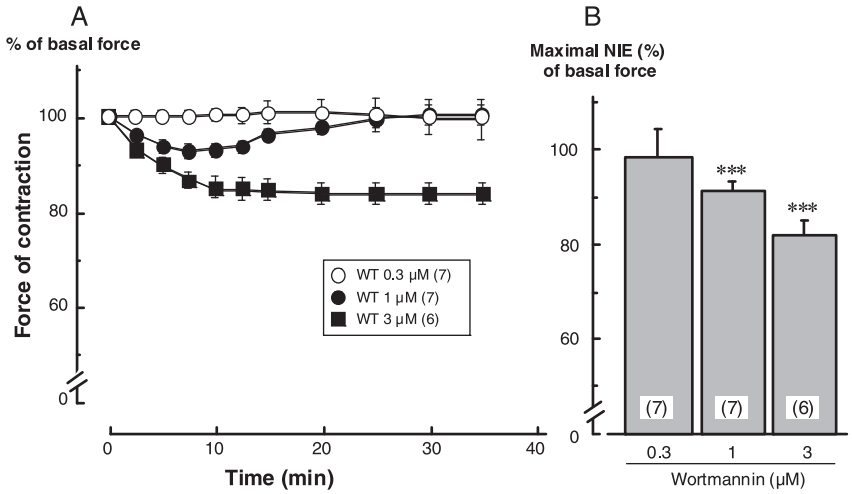

Fig. 1. Effects of wortmannin on the basal force of contraction in isolated canine ventricular myocardium. A: The time course of the NIE induced by wortmannin (WT) at concentrations of 1 and 3 μM. B: The maximal NIE induced by wortmannin. Values presented are means ± S.E.M.; average basal force of contraction prior to the administration of the drug was 6.13 ± 1.29 mN/mm^2 (n = 20). Numbers in parentheses indicate the numbers of muscle preparations. ***$P<0.001$ *vs* the basal force of contraction prior to administration of wortmannin.

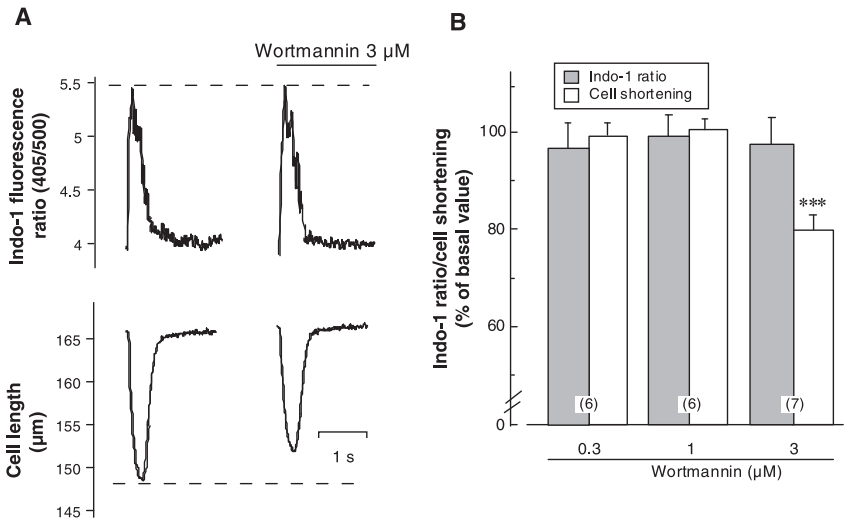

Fig. 2. Effects of wortmannin on the cell shortening and Ca^{2+} transients in isolated canine ventricular myocytes. A: Actual tracings of effects of 3 μM wortmannin in a myocyte. Individual tracings were obtained by means of signal averaging of five successive signals. Upper tracings: indo-1 fluorescence ratio and lower tracings: cell shortening. B: Summarized data of the effects of wortmannin at 0.3, 1, and 3 μM determined at the maximal levels of NIE. Baseline values prior to the administration of wortmannin were 0.78 ± 0.19 (indo-1 ratio) and 10.9 ± 2.4 μm (n = 19), respectively. Numbers in parentheses indicate the numbers of cells. ***$P<0.001$ *vs* the baseline values.

contractility, cell shortening, and Ca^{2+} transients. As shown in Fig. 1, wortmannin at concentrations of 1 μM and higher significantly inhibited the basal force of contraction (0.3 μM: 99.5 ± 3.95%, n = 7; 1 μM: 92.6 ± 1.49%, n = 7, P<0.001; 3 μM: 83.3 ± 2.67%, n = 6; P<0.001) in isolated canine ventricular trabeculae.

In canine ventricular myocytes (Fig. 2), wortmannin induced a significant decrease in cell shortening at 3 μM (0.3 μM: 99.7 ± 2.23%, n = 6; 1 μM: 101.8 ± 3.39%, n = 6; 3 μM: 78.3 ± 4.89%, n = 7, P<0.001), but it did not affect indo-1 ratio (0.3 μM: 93.9 ± 6.94%, n = 6; 1 μM: 99.6 ± 6.57%, n = 6; 3 μM: 97.1 ± 5.32%, n = 7), implying that the reduction in cell shortening may be due to a decrease in Ca^{2+} sensitivity.

Influence of wortmannin on the ET-1–induced PIE and increase in Ca^{2+} transients

ET-1 alone did not induce PIE (10 nM: 102.1 ± 0.54%, n = 6; 100 nM: 99.5 ± 1.91%, n = 6), but in the presence of NE at 1 nM, ET-1 elicited a sustained PIE (10 nM: 118.4 ± 2.49%, n = 8; 100 nM: 158.0 ± 7.77%, n = 6) as shown in Fig. 3A, a and b, with 100 nM ET-1. Wortmannin at 1 μM significantly inhibited the PIE of 10 nM ET-1 in the presence of 1 nM NE (103.2 ± 2.85%; n = 5); and it partially inhibited the PIE of 100 nM ET-1 (0.1 μM wortmannin: 156.1 ± 5.25%, n = 5; 1 μM wortmannin: 120.4 ± 5.91%; n = 5, P<0.001) (Fig. 3Ac; Fig. 4D). ET-1 elicited a transient NIE prior to sustained PIE (Fig. 3: A, B, and C), which was not affected by wortmannin (Fig. 3C).

Similar results were observed in single ventricular myocytes (Fig. 4). ET-1 at 10 nM alone did not

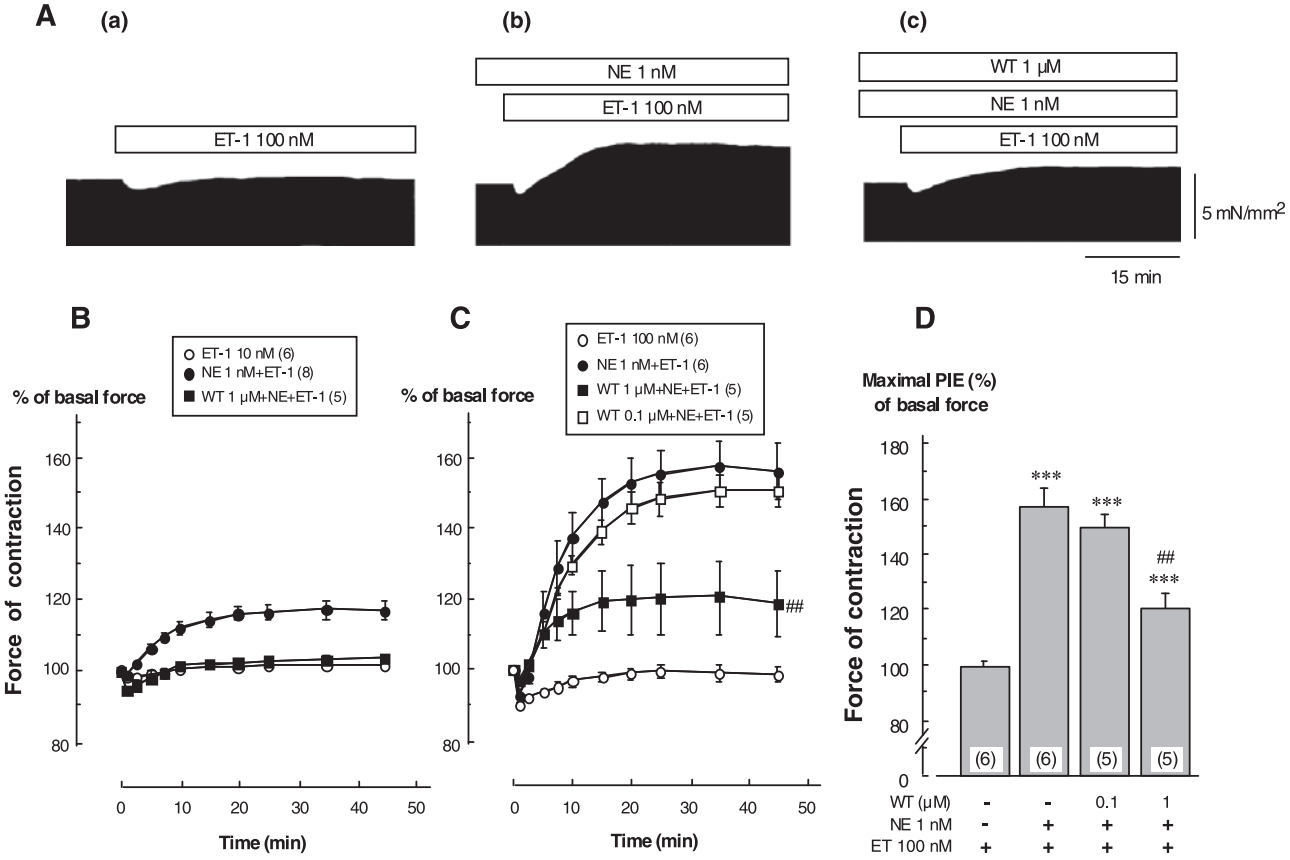

Fig. 3. Influence of wortmannin on the ET-1-induced PIE in isolated canine ventricular trabeculae. A: Actual tracings of isometric contractions: (a) ET-1 at 100 nM alone; (b) ET-1 at 100 nM in the presence of 1 nM NE; and (c) influence of 1 μM wortmannin (WT) on the PIE of 100 nM ET-1 in the presence of 1 nM NE. B: Time courses of inotropic responses to ET-1 at 10 nM: influence of 1 μM wortmannin on the PIE of 10 nM ET-1 in the presence of 1 nM NE. C: Time courses of inotropic responses to 100 nM ET-1: influence of 0.1 and 1 μM wortmannin on the PIE of 100 nM ET-1 in the presence of 1 nM NE. D: Summarized data of C determined at the maximal levels of ET-1–induced PIE. Basal force of contraction in this series of experiments was 8.8 ± 1.56 mN/mm^2 (n = 30). Numbers in parentheses indicate the numbers of muscle preparations. Values presented are means ± S.E.M.; where not shown, the S.E.M. is smaller than the symbol. Experiments were carried out in the presence of 300 nM prazosin. ***P<0.001 *vs* ET-1 alone, $^{\#\#}P$<0.001 *vs* NE + ET-1 in the absence of wortmannin.

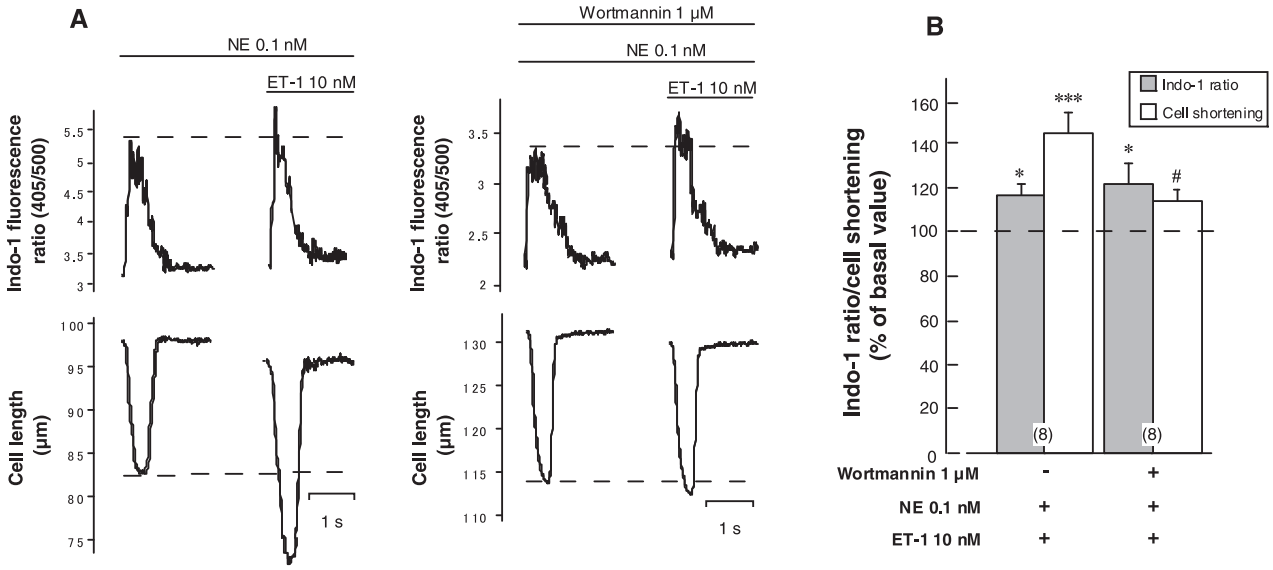

Fig. 4. Influence of 1 μM wortmannin on the 10 nM ET-1–induced increase in Ca^{2+} transients and cell shortening in the presence of 0.1 nM NE in isolated canine ventricular myocytes. A: Actual tracings of influence of wortmannin on the effects of ET-1: left panel in the absence and right panel in the presence of 1 μM wortmannin in each myocyte. Upper tracings: indo-1 fluorescence ratio and lower tracings: cell shortening. Individual tracings were obtained by means of signal averaging of five successive signals. B: Summarized data of panel A determined at the maximal levels of the ET-1–induced responses. Baseline values prior to the administration of the drugs were 0.69 ± 0.11 (indo-1 ratio) and 13.0 ± 1.35 μm (n = 16), respectively. Numbers in parentheses indicate the numbers of cells. Experiments were carried out in the presence of 300 nM prazosin. *P<0.05, ***P<0.001 *vs* the respective baseline values; $^{\#}P$<0.05 *vs* NE + ET-1 in the absence of wortmannin.

significantly affect the baseline indo-1 ratio and cell shortening (97.9 ± 10.05% and 102.3 ± 6.58% of the baseline values, respectively). However, in the presence of NE at 0.1 nM, which did not affect significantly Ca^{2+} transients and cell shortening (data not shown), ET-1 induced a significant increase in cell shortening (145.3 ± 8.05% of baseline level) associated with a small but significant increase in the indo-1 ratio (117.3 ± 5.83% of the baseline level) (Fig. 4A, left panel; Fig. 4B). Wortmannin inhibited the ET-1–induced increase in cell shortening to 115.3 ± 2.61% (n = 8, P<0.001) without affecting the ET-1–induced increase in Ca^{2+} transients (121.6 ± 4.58%, n = 8, P>0.05) (Fig. 4A, right panel; Fig. 4B), suggesting that wortmannin inhibited the increase in Ca^{2+} sensitivity in response to ET-1.

Influence of wortmannin on the PIE of NE induced by β-adrenoceptor stimulation

Wortmannin did affect the CRC for the PIE of NE (Fig. 5; Table 1). Neither the pD_2 values [$-\log_{10}$ 50% effective concentration (EC_{50})] nor the maximal response to NE was influenced by wortmannin in isolated canine ventricular trabeculae.

In single ventricular myocytes (Fig. 6), 30 nM NE increased significantly the cell shortening and indo-1 ratio (cell shortening: 186.7 ± 17.9%; n = 7, P<0.001;

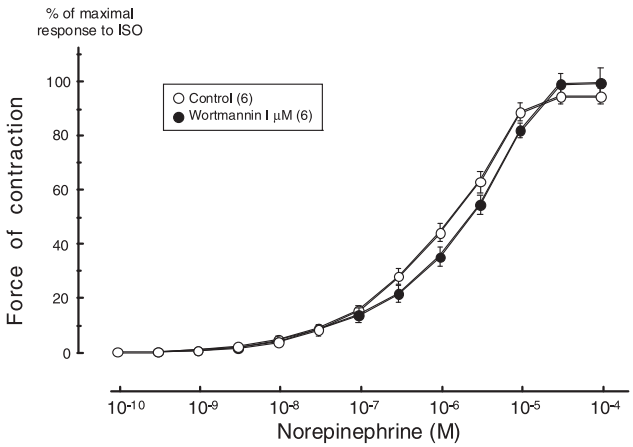

Fig. 5. Influence of 1 μM wortmannin (WT) on the CRC for the NE-induced PIE mediated by activation of β-adrenoceptors in isolated canine ventricular trabeculae. Ordinate: the PIE expressed as a percentage of the maximal response to ISO in the absence of WT; abscissa: molar concentration of norepinephrine (NE). Open circles: in the absence of WT and closed circles: in the presence of 1 μM WT. Basal force of contraction prior to the administration of NE and the ISO_{max} were 3.88 ± 0.56 mN/mm^2 and 13.4 ± 1.70 mN/mm^2 (n = 12 each), respectively. Numbers in parentheses indicate the numbers of muscle preparations. Values presented are means ± S.E.M.; where not shown, S.E.M. is smaller than the symbol. Experiments were carried out in the presence of 300 nM prazosin.

Table 1. Effects of 1 μM wortmannin on pD_2 values for NE and the maximal response to NE induced by β-adrenoceptor activation in canine ventricular myocardium

Wortmannin (μM)	n	pD_2 values	*P*	Maximal response (% of ISO_{max})	*P*
0	7	5.82 ± 0.08	—	94.4 ± 2.9	—
1	6	5.63 ± 0.06	n.s.	99.4 ± 5.2	n.s.

n indicates the number of muscle preparations. The values presented are means ± S.E.M. ISO_{max}: the maximal response to isoproterenol and n.s.: not significantly different from the control.

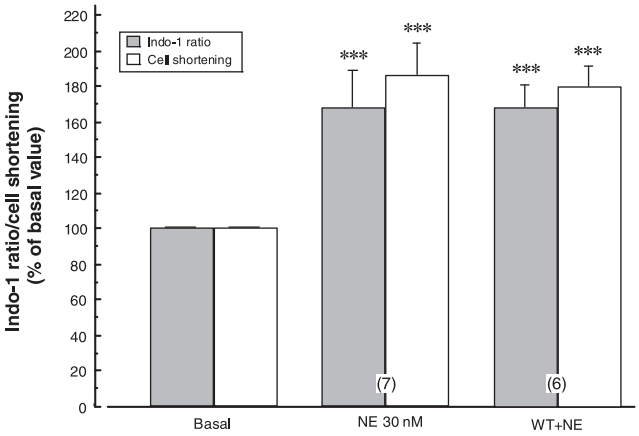

Fig. 6. Influence of 1 μM wortmannin (WT) on the 30 nM NE–induced increase in cell shortening and Ca^{2+} transients mediated by activation of β-adrenoceptors in isolated canine ventricular myocytes. Baseline values prior to the administration of NE were 0.72 ± 0.09 (indo-1 ratio) and 11.0 ± 1.63 μm (n = 12 each), respectively. Numbers in parentheses indicate the numbers of cells. ***P<0.001 *vs* the respective baseline values. Experiments were carried out in the presence of 300 nM prazosin.

indo-1 ratio: 167.3 ± 20.7% of the respective baseline levels; n = 7, P<0.001). Wortmannin at 1 μM did not significantly affect the 30 nM NE-induced increases in cell shortening and Ca^{2+} transients (199.9 ± 10.7% and 168.0 ± 11.9% of baseline values, n = 6, respectively).

Influence of wortmannin on the NIE of ET-1 in the presence of high concentrations of NE

In the presence of 100 nM NE, ET-1 produced a long-lasting NIE as indicated by the actual tracings shown in the left panel of Fig. 7A. NE at 100 nM elicited a PIE that reached the peak level 10 – 15 min after the addition, and this was sustained for 60 min, although it declined spontaneously to 86.7 ± 4.2% (n = 10) of the maximum response after 60 min (P<0.05). ET-1 at 10 nM elicited a prominent sustained NIE when it was administered 15 min after the application of NE as indicated in the actual tracings shown in the left panel of Fig. 7A. Time courses of the NIE of ET-1 are shown in Fig. 7B, and the summarized data determined at the maximal levels of NIE of ET-1 are presented in Fig. 7C. At 1 and 3 μM, wortmannin had no effect on the NIE of ET-1 in the presence of 100 nM NE: in the presence of wortmannin at 1 and 3 μM, the NIE was 34.3 ± 13.9% (n = 5) and 37.0 ± 6.7% (n = 5) of the maximum response to NE, which were not significantly different from the control response of 37.0 ± 4.3% (n = 10).

Discussion

Wortmannin at 1 μM suppressed the PIE of ET-1 induced by cross-talk with NE at the subthreshold concentration of 0.1 nM without affecting the increase in Ca^{2+} transients (Fig. 4). This finding is consistent with the inhibitory action of wortmannin on the PIE of ET-1 in rabbit ventricular myocardium (20), although the upstream signaling process is different between both species since in the rabbit, ET-1 alone is capable of inducing the PIE (6 – 9), while in the dog, it requires cross-talk with NE.

In the canine ventricular myocardium, the PIE of ET-1 induced by cross-talk with NE is susceptible to the β-adrenoceptor–blocking agent timolol and the PKA inhibitor H-89 as well as PKC inhibitors, staurosporine, H-7, and GF 109203X (4). These observations imply that the activations of both PKA induced by cAMP generated by weak β-adrenoceptor stimulation and PKC activated by diacylglycerol (DAG) produced by ET_A-receptor stimulation are required for induction of the PIE (4). By contrast, in the rabbit ventricular myocardium, the PIE elicited by ET-1 alone is not susceptible to β-adrenoceptor–blocking agent, while likewise inhibitable with PKC inhibitor (1, 8). The present results indicate that the mechanism of myofilament Ca^{2+} sensitization induced by ET-1 is identical in that activation of MLCK may be involved in both species, which is susceptible to wortmannin at a concentration of 1 μM. These findings are consistent with those of Rossmanith et al. that the PIE of ET-1 may result from an increased Ca^{2+} sensitivity related to an increase in MLC-2 phosphorylation in cardiac muscle (10).

It has been shown that ET-1, angiotensin II (AT_1-receptor stimulation), and phenylephrine (α_1-adrenoceptor stimulation) share the common signaling pathway coupled to heterotrimeric G_q proteins resulting in activation of PKC and MLCK in the heart (10, 15, 16). Namely, activation of G_q proteins leads to generation of DAG that stimulates PKC activity to lead to phosphorylation of both troponin I (13, 23, 24) and MLC-2 (13, 23) in cardiac myocytes. It is reported that phosphorylation of a serine residue in troponin I is associated with a decrease in Ca^{2+} sensitivity of the myofilament

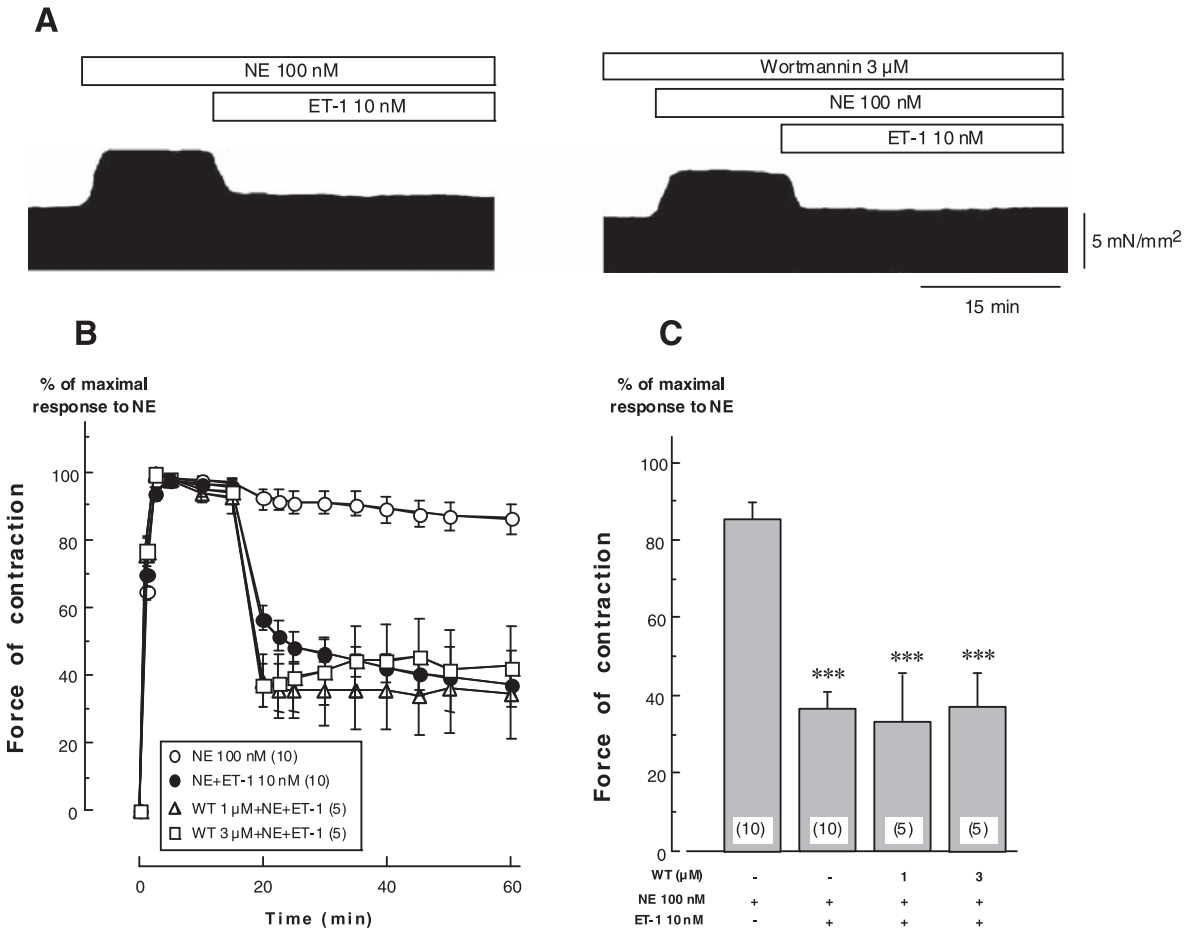

Fig. 7. Influence of wortmannin (WT) on the NIE of 10 nM ET-1 in the presence of 100 nM NE in isolated canine ventricular trabeculae. A: Actual tracings of isometric contractions: the NIE of 10 nM ET-1 in the presence of 100 nM NE (left panel) and influence of pretreatment with WT at 3 μM on the NIE of 10 nM ET-1 in the presence of 100 nM NE (right panel). B: Time courses of the NIE of 10 nM ET-1: influence of 1 and 3 μM WT on the NIE induced by 10 nM ET-1 in the presence of 100 nM NE. C: Summarized data of panel B determined at the maximal levels of ET-1–induced NIE. The maximum response to 100 nM NE before the addition of ET-1 was assigned to 100% for each preparation, and the changes in force of contraction are expressed as a percentage of the maximum response. Basal force of contraction and the maximum increase in force induced by 100 nM NE were 4.45 ± 0.50 and 7.01 ± 0.63 mN/mm^2 ($n = 10$ each), respectively. Values presented are means $\pm$ S.E.M. Numbers in parentheses indicate the numbers of muscle preparations. ***P<0.001 *vs* the response to NE alone. Experiments were carried out in the presence of 300 nM prazosin.

MgATPase (23), while phosphorylation of MLC-2 is associated with an increase in the ATPase activity and the Ca^{2+} sensitivity for force production (13, 23). In transgenic mice over-expressing non-phosphorylatable MLC-2, there was no shift of the myofibrillar Ca^{2+} sensitivity after treatment with MLCK, whereas the Ca^{2+} sensitivity was increased by MLCK in non-transgenic mice (25). Taken together, these observations suggest that the phosphorylation of MLC-2 may play an important role in the increase in myofilament Ca^{2+} sensitivity in cardiac muscle. It is also reported that the intracellular alkalization due to the PKC-induced activation of Na^+/H^+ exchanger (NHE) may be responsible for the increase in Ca^{2+} sensitivity (26 – 28) through pH-dependent MLCK activation (29). However, the net effect of PKC activation may be due to differential activation of different PKC isozymes that phosphorylate troponin I because it is reported that the Ca^{2+} sensitivity is increased by PKCε activation (30), while decreased by activation of PKCβ (31).

In the presence of higher concentrations of NE (100 – 1,000 nM), ET-1 induced a sustained NIE by antagonizing the PIE of NE mediated by β-adrenoceptors in a concentration-dependent manner in the canine ventricular myocardium (ref. 2, Fig. 7). Since the sustained NIE of ET-1 is susceptible to the phosphatase inhibitor cantharidin (4), dephosphorylation of functional proteins (phosphorylated previously by β-adrenoceptor stimula-

tion) may be involved in the sustained NIE of ET-1. The findings that the sustained NIE of ET-1 is inhibitable with pertussis toxin, the guanylyl cyclase inhibitor LY83583, and the PKG inhibitor KT5832, indicate that the cGMP/PKG signaling pathway activated by ET_A-receptor stimulation may play a crucial role in induction of the NIE.

The sustained NIE of ET-1 is associated with a concurrent decrease in Ca^{2+} transients, but not with detectable alteration of Ca^{2+} sensitivity, indicating that ET-1 stimulates the cGMP/PKG-mediated signaling process to lead to the NIE, which is unmasked in the presence of strong β-adrenoceptor stimulation (2, 4).

Neither the sustained NIE (Fig. 7) nor the transient NIE (Fig. 3) of ET-1 was affected by wortmannin at 1 μM that suppressed significantly the increase in Ca^{2+} sensitivity induced by ET-1. Furthermore, the PIE and increase in Ca^{2+} transients induced by NE through β-adrenoceptor stimulation are unaffected by wortmannin at 1 μM (Figs. 5 and 6). These observations indicate that the inhibitory effect of wortmannin on the ET-1–induced sustained PIE may be ascribed to inhibition of MLCK, but not due to a non-specific action of the compound. Nonetheless, contributions of other mechanisms are not completely excluded because wortmannin possesses various actions; thus determination of whether other mechanisms may be involved requires the use of other inhibitors.

Wortmannin in the nM concentration range (IC_{50} = 3 nM) inhibits cellular responses through the inhibition of PI3-K in non-cardiac cells (17, 32), but it has no effect on baseline levels of Ca^{2+} transients and cell shortening and the ET-1-induced effect up to 300 nM in the canine ventricular myocardium. Therefore, it is postulated that the effect of wortmannin on PI3-K does not contribute to the inhibitory effect of the compound on the ET-1–induced Ca^{2+} sensitization under the present experimental condition.

In summary, the current findings indicate that in canine ventricular myocardium, wortmannin in the μM concentration range inhibits the PIE of ET-1 induced by cross-talk with a subthreshold concentration of NE without affecting the amplitude of Ca^{2+} transients. On the other hand, wortmannin at the same concentration does not affect the PIE of NE induced by β-adrenoceptor stimulation, the sustained NIE of ET-1 elicited by activation of phosphatase via the cGMP/PKG signaling pathway, and transient NIE, an indication that the inhibitory effect of wortmannin may be exerted selectively on the ET-1–induced PIE by suppression of ET-1–induced myofilament Ca^{2+} sensitization due to MLKC inhibition in canine ventricular myocardium.

Acknowledgment

This work was supported in part by a Grant-in-Aid for Scientific Research (B) (No. 16390064) from the Ministry of Education, Culture, Sports, Science, and Technology, Japan.

References

1 Takanashi M, Endoh M. Characterization of positive inotropic effect of endothelin on mammalian ventricular myocardium. Am J Physiol. 1991;261:H611–H619.
2 Zhu Y, Yang HT, Endoh M. Negative chronotropic and inotropic effects of endothelin isopeptides in mammalian cardiac muscle. Am J Physiol. 1997;273:H119–H127.
3 Chu L, Endoh M. Biphasic inotropic response to endothelin-1 in the presence of various concentrations of norepinephrine in canine ventricular myocardium. J Cardiovasc Pharmacol. 2000; 36 Suppl 2:S9–S14.
4 Chu L, Takahashi R, Norota I, Miyamoto T, Takeishi Y, Ishii K, et al. Signal transduction and Ca^{2+} signaling in contractile regulation induced by crosstalk between endothelin-1 and norepinephrine in dog ventricular myocardium. Circ Res. 2003;92:1024–1032.
5 Chu L, Zhang JX, Norota I, Endoh M. Receptor subtypes mediating the inotropic effects and Ca^{2+} signaling induced by endothelin-1 through crosstalk with norepinephrine in canine ventricular myocardium. J Pharmacol Sci. 2005;97:417–428.
6 Fujita S, Endoh M. Effects of endothelin-1 on $[Ca^{2+}]_i$-shortening trajectory and Ca^{2+} sensitivity in rabbit single ventricular cardiomyocytes loaded with indo 1-AM: comparison with the effects of phenylephrine and angiotensin II. J Card Fail. 1996;2 Suppl: S45–S57.
7 Yang HT, Sakurai K, Sugawara H, Watanabe T, Norota I, Endoh M. Role of Na^+/Ca^{2+} exchange in endothelin-1-induced increases in Ca^{2+} transient and contractility in rabbit ventricular myocytes: pharmacological analysis with KB-R7943. Br J Pharmacol. 1999;126:1785–1795.
8 Talukder MA, Norota I, Sakurai K, Endoh M. Inotropic response of rabbit ventricular myocytes to endothelin-1: difference from isolated papillary muscles. Am J Physiol. 2001;281:H596–H605.
9 Wang H, Endoh M. Chelerythrine and genistein inhibit the endothelin-1-induced increase in myofilament Ca^{2+} sensitivity in rabbit ventricular myocytes. Eur J Pharmacol. 2001;424:91–96.
10 Rossmanith GH, Hoh JF, Turnbull L, Ludowyke RI. Mechanism of action of endothelin in rat cardiac muscle: cross-bridge kinetics and myosin light chain phosphorylation. J Physiol. 1997;505:217–227.
11 Endoh M, Fujita S, Yang HT, Talukder MA, Maruya J, Norota I. Endothelin: receptor subtypes, signal transduction, regulation of Ca^{2+} transients and contractility in rabbit ventricular myocardium. Life Sci. 1998;62:1485–1489.
12 Shimokawa H, Seto M, Katsumata N, Amano M, Kozai T, Yamawaki T, et al. Rho-kinase-mediated pathway induces enhanced myosin light chain phosphorylations in a swine model of coronary artery spasm. Cardiovasc Res. 1999;43:1029–1039.
13 Clement O, Puceat M, Walsh MP, Vassort G. Protein kinase C enhances myosin light-chain kinase effects on force development and ATPase activity in rat single skinned cardiac cells. Biochem J. 1992;285:311–317.
14 Morano I. Tuning the human heart molecular motors by myosin light chains. J Mol Med. 1999;77:544–555.

15 Andersen GGO, Qvigstad E, Schiander I, Aass H, Osnes JB, Skomedal T. α_1-AR-induced positive inotropic response in heart is dependent on myosin light chain phosphorylation. Am J Physiol. 2002;283:H1471–H1480.
16 Grimm M, Haas P, Willipinski-Stapelfeldt B, Zimmermann WH, Rau T, Pantel K, et al. Key role of myosin light chain (MLC) kinase-mediated MLC2a phosphorylation in α_1-adrenergic positive inotropic effect in human atrium. Cardiovasc Res. 2005;65:211–220.
17 Yano H, Nakanishi S, Kimura K, Hanai N, Saitoh Y, Fukui Y, et al. Inhibition of histamine secretion by wortmannin through the blockade of phosphatidylinositol 3-kinase in RBL-2H3 cells. J Biol Chem. 1993;268:25846–25856.
18 Burdyga TV, Wray S. The effects of inhibition myosin light chain kinase by wortmannin on intracellular [Ca^{2+}], electrical activity and force in phasic smooth muscle. Pflügers Arch-Eur J Physiol. 1998;436:801–803.
19 Longbottom ER, Lukas MJM, Kupittayanant S, Badrick E, Shimigol T, Wray S. The effects of inhibiting myosin light chain kinase on contraction and calcium signaling in human and rat myometrium. Pflügers Arch-Eur J Physiol. 2000;440:315–321.
20 Chu L, Endoh M. Wortmannin inhibits the myofilament Ca^{2+} sensitization induced by endothelin-1. Eur J Pharmacol. 2005;507:135–143.
21 Watanabe T, Endoh M. Antiadrenergic effects of the endothelin-1 on the L-type Ca^{2+} current in dog ventricular myocytes. J Cardiovasc Pharmacol. 2000;36:344–350.
22 Grynkiewicz G, Poenie M, Tsien RY. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. J Biol Chem. 1985;260:3440–3450.
23 Noland TA Jr, Gou X, Raynor RL, Jideama NM, Averyhart-Fullard V, Solaro RJ, et al. Cardiac troponin I mutants. Phosphorylation by protein kinases C and A and regulation of Ca^{2+}-stimulated MgATPase of reconstituted actomyosin S-1. J Biol Chem. 1995;270:25445–25454.
24 Kanaya N, Gable B, Murray PA, Damron DS. Propofol increases phosphorylation of troponin I and myosin light chain 2 via protein kinase C activation in cardiomyocytes. Anesthesiology. 2003;98:1363–1371.
25 Sanbe A, Fewell JG, Gulick J, Osinska H, Lorenz J, Greg HD, et al. Abnormal cardiac structure and function in mice expressing nonphosphorylatable cardiac regulatory myosin light chain 2. J Biol Chem. 1999;274:21085–21094.
26 Talukder MA, Endoh M. Pharmacological differentiation of synergistic contribution of L-type Ca^{2+} channels and Na^+/H^+ exchange to the positive inotropic effect of phenylephrine, endothelin-3 and angiotensin II in rabbit ventricular myocardium. Naunyn Schmiedebergs Arch Pharmacol. 1997;355:87–96.
27 Wang H, Sakurai K, Endoh M. Pharmacological analysis by HOE642 and KB-R9032 of the role of Na^+/H^+ exchange in the endothelin-1-induced Ca^{2+} signalling in rabbit ventricular myocytes. Br J Pharmacol. 2000;131:638–644.
28 Zolk O, Munzel F, Eschenhagen T. Effects of chronic endothelin-1 stimulation on cardiac myocyte contractile function. Am J Physiol. 2004;286:H1248–H1257.
29 Shrode LD, Klein JD, O'Neill WC, Putnam RW. Shrinkage-induced activation of Na^+/H^+ exchange in primary rat astrocytes: role of myosin light-chain kinase. Am J Physiol. 1995;269:C257–C266.
30 Takeishi Y, Ping P, Bolli R, Kirkpatrick DL, Hoit BD, Walsh RA. Transgenic overexpression of constitutively active protein kinase Cε causes concentric cardiac hypertrophy. Circ Res. 2000;86:1218–1223.
31 Takeishi Y, Chu G, Kirkpatrock DM, Li Z, Wakasaki H, Kranias EG, et al. In vivo phosphorylation of cardiac troponin I by protein kinase Cβ2 decreases cardiomyocyte calcium responsiveness and contractility in transgenic mouse hearts. J Clin Invest. 1998;102:72–78.
32 Powis G, Bonjouklian R, Berggren MM, Gallegos A, Abraham R, Ashendel C, et al. Wortmannin, a potent and selective inhibitor of phosphatidylinositol-3-kinase. Cancer Res. 1994;54:2419–2423.