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Wortmannin inhibits the myofilament Ca^{2+} sensitization induced by endothelin-1

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Abstract

Endothelin-1 induces a positive inotropic effect due to a combination of an increase in Ca^{2+} transients and myofilament Ca^{2+} sensitivity in rabbit ventricular myocardium. We carried out the experiments to examine the potential contribution of myosin light chain kinase to the Ca^{2+} sensitization induced by endothelin-1 by use of wortmannin that inhibits myosin light chain kinase at high concentrations (IC_{50}=200 nM). Wortmannin at 3 μM suppressed the basal force of contraction, but did not affect the positive inotropic effect mediated by β-adrenoceptors. Wortmannin at 1 and 3 μM markedly inhibited the positive inotropic effect of endothelin-1, but did not affect the increase in Ca^{2+} transients elicited by endothelin-1. The present findings imply that the increase in myofilament Ca^{2+} sensitivity induced by endothelin-1 may be in part due to activation of myosin light chain kinase in rabbit ventricular myocardium.

Keywords: Inotropic effect; Ca^{2+} transient; Endothelin-1; Wortmannin; Rabbit cardic myocyte

1. Introduction

Endothelin-1 is a potent vasoactive peptide produced by various types of cardiovascular cells, including endothelial cells, vascular smooth muscle, and myocardial cells (Yanagisawa et al., 1988). Endothelin receptors belong to the G_q-coupled receptors and lead to acceleration of phosphoinositide hydrolysis via activation of phospholipase Cβ. In turn, phospholipase Cβ activation results in production of inositol 1,4,5-trisphosphate which releases Ca^{2+} from intracellular stores, and diacylglycerol, which activates protein kinase C, thus contributes to the contractile regulation induced by endothelin-1, angiotensin II, and α_1-adrenergic receptor agonists in the heart (Endoh, 1997). Endothelin-1 elicits a pronounced positive inotropic effect in the mammalian cardiac muscle of most species, including rabbits, rats, guinea pigs, ferrets, and humans (Takanashi and Endoh, 1991; Qiu et al., 1992).

The positive inotropic effect of endothelin-1 is due to combination of the modest increase in intracellular Ca^{2+} transients and an increase in myofilament Ca^{2+} sensitivity, but the subcellular mechanisms have not been fully elucidated (Yang et al., 1999; Talukder et al., 2001). The increase in Ca^{2+} sensitivity of contractile proteins in intact myocytes has been partially explained by intracellular alkalinization and/or increased phosphorylation of the myofilaments (Rossmanith et al., 1997; Endoh et al., 1998). In smooth muscle cells, myosin light chain kinase and Rho-kinase act in concert to phosphorylate myosin light chain-2 (Somlyo and Somlyo, 1994).

Myosin light chain kinase has been shown to phosphorylate myosin light chain-2 and increase myofilament sensitivity to Ca^{2+} in cardiac skinned fibers (Clement et al., 1992; Morano, 1999). Furthermore, endothelin-1 increases myosin light chain phosphorylation in cardiac muscle (Rossmanith et al., 1997), and the positive inotropic effect of α_1-adrenoceptor stimulation is

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dependent on myosin light chain kinase phosphorylation (Andersen et al., 2002). However, involvement of myosin light chain kinase in the inotropic response to endothelin-1 in intact myocardium has remained elusive. The fungal metabolite wortmannin has become a popular tool for unraveling the role of myosin light chain kinase, since it is a potent inhibitor of the enzyme at *micromolar* concentrations and the EC_{50} value for the inhibition is 200 nM (Yano et al., 1993).

The positive inotropic effect of endothelin-1 varies widely among mammalian species, and is most pronounced in rabbits among the mammalian species examined to date (Takanashi and Endoh, 1991; Yomogida et al., 2004). Thus, in the present study, we used wortmannin at 1–3 μM to elucidate the role of myosin light chain kinase inhibition in the inotropic response to endothelin-1 in the rabbit ventricular myocardium, since these concentrations have been shown to be effective for myosin light chain kinase inhibition in smooth muscle tissues (Burdyga and Wray, 1998; Longbottom et al., 2000). For comparison, the effects of wortmannin on the positive inotropic effect of isoproterenol mediated by β-adrenoceptors were also investigated. The experiments were carried out in both rabbit papillary muscles and indo-1-loaded ventricular myocytes, since the inotropic effect of endothelin-1 differs quantitatively between these preparations (Talukder et al., 2001).

2. Methods

The current study involving experimental animals conformed to our institutional standards, and was performed in accordance with the National Institutes of Health (NIH publication) *Guide for the Care and Use of Laboratory Animals*. Approval for the animal experiments was obtained from the Committee for Animal Experimentation, Yamagata University School of Medicine, prior to the experiments, and the study was also carried out in accordance with the Declaration of Helsinki.

2.1. Isolation of ventricular myocytes from rabbit heart

Rabbit ventricular myocytes were isolated by the Langendorff procedure with a slight modification as described previously (Fujita and Endoh, 1996). The heart was perfused with nominally Ca^{2+}-free HEPES-Tyrode solution containing 0.6 mg/ml collagenase (Type II; Worthington Biochemical, Freehold, NJ, USA) and 0.1 mg/ml protease (Type XIV; Sigma, St. Louis, MO, USA), and the dispersed cells were then filtered through a nylon mesh (200 μm). The Ca^{2+} concentration was gradually increased to 1.8 mM in a stepwise manner, and the cells were allowed to stabilize at least for 1 h at room temperature (25 °C).

2.2. Indo-1 loading, cell superfusion, and electrical stimulation

All the experimental procedures were carried out at room temperature (25 °C). Myocytes were loaded with indo-1/AM (Dojin Chemical, Kumamoto, Japan) an acetoxymethyl ester form of a Ca^{2+} fluorescent probe, by incubation in 5 μM indo-1/AM. After loading, they were centrifuged at $5\times g$ for 1 min, layered onto a superfusion chamber placed on the stage of an inverted microscope (Diaphot TMD300; Nikon, Tokyo, Japan) and allowed to settle for 15 min. Continuous superfusion was then initiated with Krebs–Henseleit bicarbonate buffer at a rate of 2 ml/min, and the myocytes were stimulated electrically with square-wave pulses of 5 ms and a voltage approximately 50% above the threshold at 0.5 Hz. The experimental protocols of drug administration were carried out after an equilibration period of 40 min.

2.3. Simultaneous measurements of cell shortening and indo-1 fluorescence ratio

Indo-1 fluorescence was excited by light from a xenon lamp (150 W) with a wavelength of 355 nm, reflected by a 380-nm long-pass dichroic mirror (Omega Optical, Brattleboro, VT, USA) and detected using a fluorescence spectrophotometer (CAM-230; Japan Spectroscopic, Tokyo, Japan). The excitation light was applied to the myocytes through a neutral density filter. The emitted fluorescence was collected by an objective lens (CF Fluor DL40; Nikon) and after passing through 380-nm long-pass dichroic mirror, it was separated by a 580-nm long-pass dichroic mirror (Omega Optical). The fluorescence light was split by a 425-nm dichroic mirror to permit simultaneous measurements at wavelengths of both 405- and 500-nm wavelengths through the band-pass filters using two separate photomultiplier tubes. The fluorescence ratio (405/500 nm) was used as an index of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) (Grynkiewicz et al., 1985).

The cell length was monitored using red light (>620 nm) through the normal bright-field illumination optics of the microscope. The cell image was collected by the 580-nm long-pass dichroic mirror. The bright-field image of the cell was projected onto the photodiode array of an edge detector (C6294-01; Hamamatsu Photonics, Hamamatsu, Japan) and scanned every 5 ms. An increase or decrease in cell shortening was considered to reflect an inotropic effect, and is often referred to as a positive or negative inotropic effect interchangeably without explanation.

2.4. Data recording and analysis

The cell length and indo-1 fluorescence signals were collected onto a Macintosh Computer using an A/D at 200 Hz. The signals were analyzed after low-pass filtering (25-Hz cutoff frequency) and averaging of five successive signals. The experimental values are presented as means±S.E.M. The

mean values between two groups were compared by Student's *t*-test for unpaired values. A *P* value of less than 0.05 was considered to indicate a statistically significant difference.

2.5. Study protocol for cell shortening and $[Ca^{2+}]_i$

The cell length was continuously measured throughout the experiments, while the indo-1 fluorescence was monitored intermittently to reduce the quenching. Wortmannin was administered 20 min before the application of endothelin-1 or isoproterenol. The α-adrenergic receptor antagonist prazosin (300 nM) was present in the superfusion chamber throughout the experiments. In the experiments with endothelin-1, β-adrenergic receptor antagonist timolol (1 μM) was also added. Experiments were carried out at room temperature, since the indo-1 ratio and cell shortening of single myocytes were more labile at the high temperature employed in papillary muscle experiments. Furthermore, since single myocytes shortened from their slack lengths without being stretched to the optimal length, they were not contracting under ideal conditions for determining contractile response to inotropic agents. Nevertheless, it has been confirmed that the responses of single myocytes to agents, such as isoproterenol, endothelin-3, and cardiotonic agents, were qualitatively and quantitatively similar to those of papillary muscles, except for endothelin-1 (Sato et al., 1998; Talukder et al., 2001; Chu et al., 2003).

2.6. Papillary muscle preparation and experimental protocols

The details of the experimental procedures used for rabbit right ventricular papillary muscle preparation were described previously (Talukder et al., 2001). Isolated papillary muscles (<1 mm in diameter, ~5 mm in length) were electrically stimulated by square-wave pulses of 5 ms and a voltage approximately 20% above the threshold at 1 Hz and 37 °C (pH 7.4). During an equilibration period of 60 min, they were stretched at a resting tension of 5 mN, and the length was adjusted to give 90% of the maximal contractile force (L_{max}). The rabbit papillary muscle preparations had average dimensions of 4.97±0.91 mm in length and 1.29±0.28 mm^2 in cross-sectional area (n=22, from 8 rabbits). Prazosin (300 nM) was present in the organ bath throughout the experiments. In the experiments with endothelin-1, timolol (1 μM) was also added. The concentration–response curve for endothelin-1 was only determined once in the absence or presence of 3 μM wortmannin in each preparation, since it was difficult to wash out endothelin-1. The maximum force was determined by application of isoproterenol. The endothelin-1-induced inotropic response was calculated as a percentage of the isoproterenol-induced maximal response (maximal force minus basal force) in individual muscles. The concentration–response curve for isoproterenol was determined successively in the absence and presence of 3 μM wortmannin in the same preparation. The maximal response to isoproterenol in the absence of wortmannin was assigned as 100%, and the increase in force induced by isoproterenol in the presence of wortmannin (maximal force minus the basal force in the presence of wortmannin) was compared with that in its absence (100%). Rabbit papillary muscle preparations contracting at 1 Hz at 37 °C have a basal force of about 4–7 mN/mm^2 and the maximal contraction induced by β-adrenergic stimulation is 20–30 mN/mm^2 (Endoh and Blinks, 1988). In the present study, the maximal response to endothelin-1 amounted to approximately 60% of the maximal response to isoproterenol, which is consistent with previous findings (Takanashi and Endoh, 1991; Talukder and Endoh, 1997; Talukder et al., 2001).

2.7. Drugs and chemicals

The following drugs were used: endothelin-1 (Peptide Institute, Osaka, Japan); prazosin hydrochloride (Pfizer Taito, Tokyo, Japan); isoproterenol hydrochloride, timolol (s-[−]-1-[t-butylamino]-3-[(4-morpholino-1,2,5-thiadiazol-3-yl) oxy]-2-propanol), wortmannin and protease Type XIV (Sigma); collagenase (Type II; Worthington Biochemical); indo-1-AM (Dojin Chemical); pentobarbital sodium (Tokyo Kasei Kogyo; Tokyo, Japan); and dimethyl sulfoxide (DMSO; Wako Pure Chemicals; Osaka, Japan). All other chemicals were of the highest analytic grade commercially available. Wortmannin and prazosin were dissolved at 10 mM in 100% DMSO solution as stock solutions, and then diluted with 0.9% NaCl to the desired concentrations. Timolol was dissolved at 10 mM in 0.9% NaCl solution as the stock solution.

3. Results

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

First, we performed experiments to elucidate the effects of wortmannin at different concentrations on the baseline contractility, cell shortening, and Ca^{2+} transients in rabbit ventricular papillary muscles and myocytes. As shown in Fig. 1, 3 μM wortmannin significantly suppressed the basal force of contraction (3 μM wortmannin: 79.04±2.63% of the basal force of contraction prior to administration, P<0.001) in rabbit ventricular papillary muscles, whereas 1 μM wortmannin had no effect (data not shown).

In rabbit ventricular myocytes, 3 μM wortmannin induced a significant decrease in the cell shortening (83.38±3.34% of the control shortening, P<0.001), but had no significant effect on the indo-1 ratio (3 μM wortmannin: 102.50±3.36% of the control ratio, P>0.05; Fig. 2), suggesting that the reduction in cell shortening may be due to a decrease in Ca^{2+} sensitivity. As shown in Fig. 2, the time courses of cell shortening and Ca^{2+} transients as detected by indo-1 ratio were also unaffected by wortmannin.

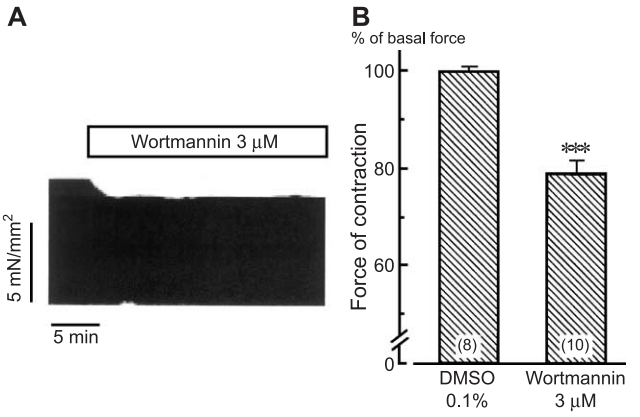

Fig. 1. Effects of wortmannin on basal force of contraction in rabbit ventricular papillary muscles. (A) Actual tracings of the negative inotropic effect induced by 3 μM wortmannin. (B) Summarized data for the effect of wortmannin. DMSO (0.1%): the solvent for wortmannin dissolution. The highest concentration of DMSO in the organ bath used to dissolve wortmannin in the current experiments was 0.03%. The basal force of contraction prior to wortmannin administration was 4.51±1.01 mN/mm^2 (n=18). The vertical bars represent the S.E.M. The numbers in parentheses indicate the numbers of muscle preparations. ***P<0.001 vs. the solvent group.

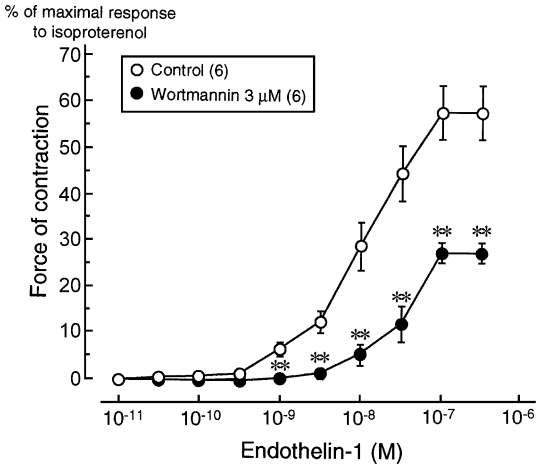

Fig. 3. Influence of 3 μM wortmannin on the concentration–response curve for the positive inotropic effect of endothelin-1 in rabbit ventricular papillary muscles. The basal force of contraction prior to administration of the drugs and the maximal response to isoproterenol were 4.86±0.28 and 34.77±12.08 mN/mm^2 (n=12 each), respectively. The numbers in parentheses indicate the numbers of muscle preparations. The vertical bars represent the S.E.M., and where they are not apparent, the S.E.M. is smaller than the symbol. Experiments were carried out in the presence of 300 nM prazosin and 1 μM timolol. **P<0.01 vs. the control response to the corresponding concentrations in the absence of wortmannin.

3.2. Influence of wortmannin on the endothelin-1-induced positive inotropic effect and increase in indo-1 fluorescence ratio

The influence of wortmannin on the positive inotropic effect of endothelin-1 was investigated in rabbit ventricular papillary muscles and myocytes. In the presence of prazosin (300 nM) and timolol (1 μM), endothelin-1 elicited a positive inotropic effect in a concentration-dependent manner over a concentration range of 0.3–100 nM in rabbit papillary muscles. Wortmannin at 3 μM shifted the concentration–response curve for endothelin-1 to the right and downward (Fig. 3). The maximum response to endothelin-1 (percentage of the maximal response to isoproterenol) and pD$_2$ value [−log (effective concentration causing 50% of the maximal response)] for endothelin-1 were significantly decreased by wortmannin. The maximal response (60.64±5.29%, n=6) and pD$_2$ value (8.01±0.07, n=6) for endothelin-1 in the control experiments were significantly decreased to 28.01±2.24%

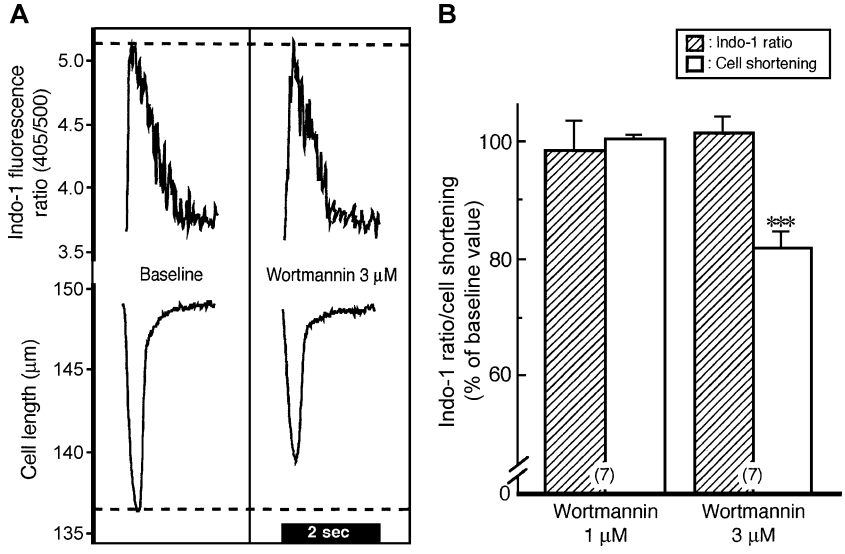

Fig. 2. Effects of wortmannin on the indo-1 fluorescence ratio and cell shortening in rabbit ventricular myocytes. (A) Actual tracings of the effects of 3 μM wortmannin. Individual tracings were obtained by signal averaging of five successive signals. *Upper tracings*: indo-1 fluorescence ratio; *lower tracings*: cell shortening. (B) Summarized data for the effects of 1 and 3 μM wortmannin. The baseline values for the indo-1 ratio and cell shortening prior to wortmannin administration were 0.91±0.15 and 16.61±1.89 μm (n=14 each), respectively. The numbers in parentheses indicate the numbers of cells. ***P<0.001 vs. the control cell shortening.

(n=6, P<0.01) and 7.53±0.11 (n=6, P<0.05), respectively, in the presence of 3 μM wortmannin.

In rabbit ventricular myocytes, 0.1 nM endothelin-1 induced a significant increase in cell shortening (171.90±8.99% of the control shortening, P<0.05), which was associated with a small but significant increase in the indo-1 ratio (120.7±7.51% of the control ratio, P<0.001; left panel in Fig. 4(A)). At 3 μM, wortmannin inhibited the endothelin-1-induced increase in cell shortening with little effect on the indo-1 ratio (right panel in Fig. 4(A)). The data regarding the influence of 0.3, 1, and 3 μM wortmannin on the 0.1 nM endothelin-1-induced effects on the cell shortening and indo-1 ratio are summarized in Fig. 4(B). Wortmannin at 0.3 μM had no effect on the endothelin-1-induced increase in cell shortening, but 1 and 3 μM wortmannin significantly inhibited the endothelin-1-induced increase in cell shortening (1 μM wortmannin: 125.52±2.46% of the control shortening, n=6, P<0.001; 3 μM wortmannin: 115.11±1.97% of the control shortening, n=7, P<0.001; Fig. 4(B)). The endothelin-1-induced

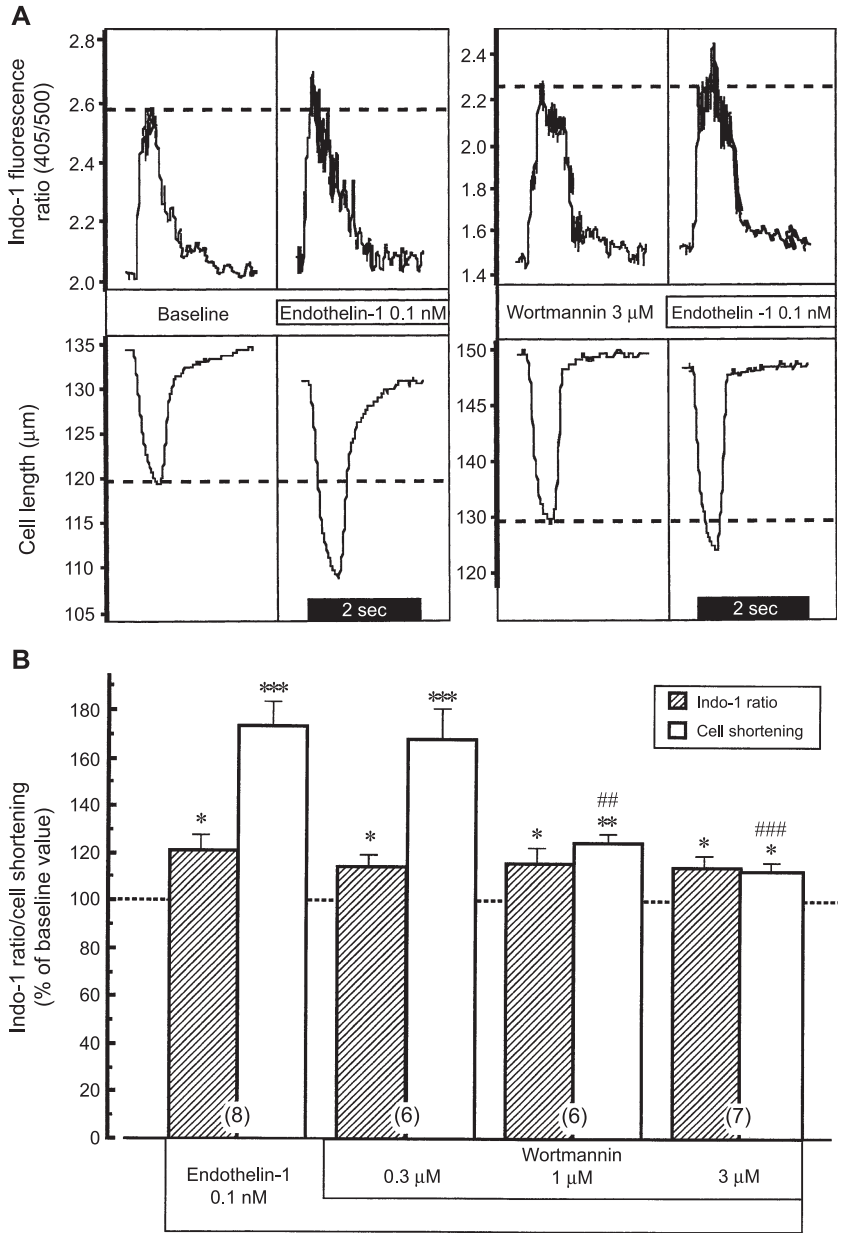

Fig. 4. Influence of wortmannin on the endothelin-1-induced increases in the cell shortening and indo-1 fluorescent ratio in rabbit ventricular myocytes. (A) Individual tracings of the effects of 0.1 nM endothelin-1 in the absence (*left panel*) and presence (*right panel*) of 3 μM wortmannin. Individual tracings were obtained by signal averaging of five successive signals. *Upper tracings*: indo-1 fluorescence ratio; *lower tracings*: cell shortening. (B) Summarized data for the effects of wortmannin at different concentrations. The baseline values for the indo-1 ratio and cell shortening prior to administration of the drugs were 1.06±0.13 and 13.19±1.89 μm (n=27 each), respectively. The numbers in parentheses indicate the number of cells. *P<0.05; **P<0.01; ***P<0.001 vs. the respective baseline values. $^{\#\#}P$<0.01; $^{\#\#\#}P$<0.001 vs. the respective values with endothelin-1 alone. The experiments were carried out in the presence of 300 nM prazosin and 1 μM timolol.

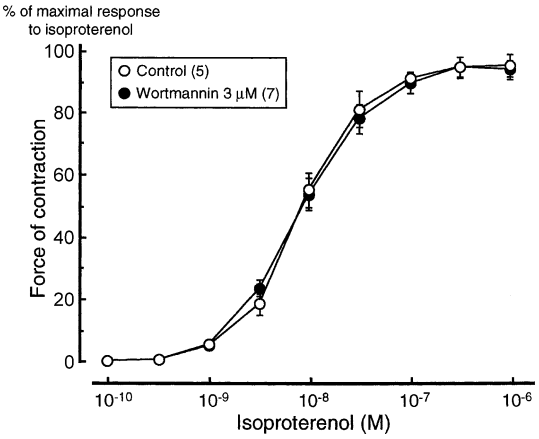

Fig. 5. Influence of 3 μM wortmannin on the concentration–response curve for the positive inotropic effect of isoproterenol in rabbit ventricular papillary muscles. The basal force of contraction prior to administration of the drugs and the maximal response to isoproterenol were 6.60±1.71 and 47.16±8.45 mN/mm^2 (n=12 each), respectively. The numbers in parentheses indicate the numbers of muscle preparations. The vertical bars represent the S.E.M., and where they are not apparent, the S.E.M is smaller than the symbol. The experiments were carried out in the presence of 300 nM prazosin.

increase in the indo-1 ratio was unaffected by wortmannin (1 μM: wortmannin 118.60±6.11% of the control ratio, n=6, P>0.05; 3 μM wortmannin: 115.80±5.17% of the control ratio, n=7, P>0.05; Fig. 4(B)).

3.3. Influence of wortmannin on the isoproterenol-induced positive inotropic effect and increase in indo-1 fluorescence ratio

Fig. 5 shows the influence of 3 μM wortmannin on the concentration–response curve for the positive inotropic effect of isoproterenol in rabbit papillary muscles. Wortmannin had no significant effect on the concentration–response curve for isoproterenol. The maximal response (94.6±3.53%, n=7) and pD$_2$ value (8.04±0.07, n=7) for isoproterenol in the presence of 3 μM wortmannin were not significantly different from the values of 97.71±3.28% (n=5, P>0.05) and 8.12±0.08 (n=5, P>0.05) in the control experiments.

In rabbit ventricular myocytes, 10 nM isoproterenol increased the cell shortening (upper panel in Fig. 6(A)). As shown in the summarized data in Fig. 6(B), 10 nM isoproterenol induced significant increases in the indo-1 ratio (165.34±12.33% of the control ratio; n=6, P<0.001) and cell shortening (164.0±7.11% of the control cell shortening; n=6, P<0.001). Wortmannin at 3 μM had no effects on the isoproterenol-induced increase in cell shortening, as shown in the actual tracings in Fig. 6(A) (lower panel) and indo-1 ratio, as shown in the summarized data in Fig. 6(B). In the presence of 3 μM wortmannin, 10 nM isoproterenol increased the indo-1 ratio to 164.90±12.95% and cell shortening to 145.24±10.60% (n=6, each) of the control signals in the absence of wortmannin (P>0.05 vs. the respective control responses, n=6 each).

4. Discussion

The important findings in the present study are that wortmannin at concentrations of 1 and 3 μM which inhibits the activity of myosin light chain kinase and PI3 kinase, suppressed the cell shortening without affecting Ca^{2+} transients in rabbit ventricular myocytes. Furthermore, at the concentrations employed, wortmannin did not alter the

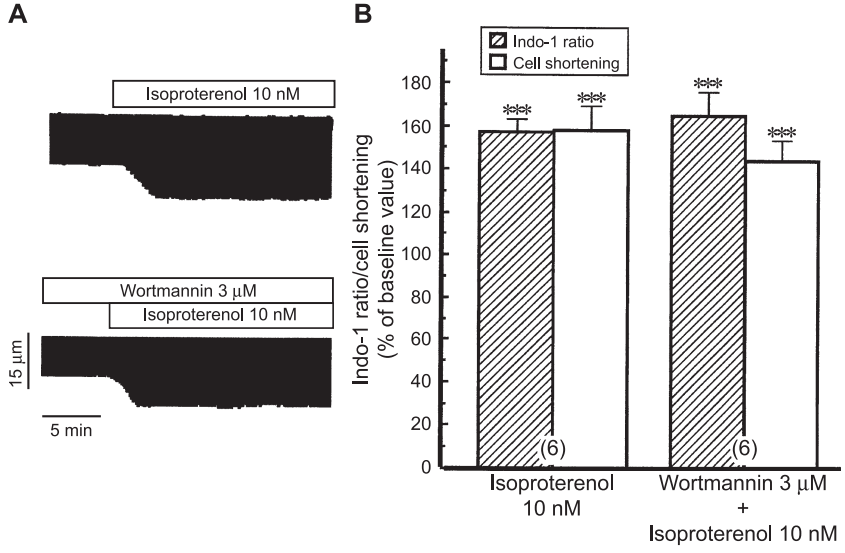

Fig. 6. Influence of 3 μM wortmannin on the isoproterenol-induced increases in the cell shortening and indo-1 fluorescence ratio in rabbit ventricular myocytes. (A) Actual tracings of the effects of 10 nM isoproterenol on the cell shortening in the absence (*upper tracings*) and presence (*lower tracings*) of 3 μM wortmannin. (B) Summarized data. The baseline values for the indo-1 ratio and cell shortening prior to administration of the drug were 0.66±0.17 and 11.39±1.19 μm (n=13 each), respectively. The numbers in parentheses indicate the number of cells. ***P<0.001 vs. the respective baseline values. The experiments were carried out in the presence of 300 nM prazosin.

effects of isoproterenol on contractility and Ca^{2+} transients under the same experimental conditions.

Although wortmannin inhibits myosin light chain kinase in vitro with high specificity compared to other inhibitors (Davies et al., 2000), it also possesses a potent inhibitory action on PI3 kinase (Yano et al., 1993). Wortmannin can inhibit various cellular responses through inhibition of PI3 kinase in the *nanomolar* concentration range (Yano et al., 1993), and has IC_{50} values of 3.0 nM for PI3 kinase inhibition, and 200 nM for myosin light chain kinase inhibition (Yano et al., 1993). The observation that 0.3 μM wortmannin was ineffective in rabbit ventricular myocytes (Fig. 4) implies that it may primarily act as a specific inhibitor of myosin light chain kinase under the current experimental conditions. The concentration of wortmannin employed in the present study was previously shown to be sufficient for myosin light chain kinase inhibition in smooth muscle cells (Burdyga and Wray, 1998; Longbottom et al., 2000).

4.1. Influence of wortmannin on endothelin-1-induced positive inotropic effect

The remarkable increase in cell shortening induced by endothelin-1 was associated with a marginal increase in Ca^{2+} transients (Fig. 4) compared with the effect of β-adrenergic stimulation (Fig. 6). These results are consistent with previous findings that the positive inotropic effect of endothelin-1 is due to the combination of an increase in myofilament Ca^{2+} sensitivity and a small but significant increase in Ca^{2+} transients (Fujita and Endoh, 1996; Yang et al., 1999; Talukder et al., 2001).

The mechanism of the increase in Ca^{2+} sensitivity has been partially ascribed to intracellular alkalinization, protein kinase C activation, and increased myosin light chain phosphorylation (Rossmanith et al., 1997; Endoh et al., 1998). The current findings that wortmannin at 1 and 3 μM suppressed the endothelin-1-induced increase in cell shortening without affecting the Ca^{2+} transients indicate that phosphorylation of the myosin light chain may play a crucial role in the increase in Ca^{2+} sensitivity induced by endothelin-1. In a previous study, it was found that endothelin-1 was 60-fold more potent in rabbit ventricular myocytes than in papillary muscles, while the potencies of the positive inotropic effect of isoproterenol, endothelin-3, angiotensin II, and levosimendan were identical in both preparations (Fujita and Endoh, 1996; Sato et al., 1998; Talukder et al., 2001). Although the reason for this difference is unknown, the effect of wortmannin was not influenced by the preparations, since at a concentration of 3 μM it suppressed the basal force, baseline cell shortening, and effects of endothelin-1 in both myocyte and papillary muscle preparations.

In smooth muscle cells, wortmannin inhibits the contractions induced by various agonists and high K^+ without changing $[Ca^{2+}]_i$ (Burdyga and Wray, 1998; Longbottom et al., 2000; Ito et al., 2004). The effect of α_1-adrenergic receptor stimulation on smooth muscle contraction is ascribed to activation of the Ca^{2+}-calmodulin-dependent myosin light chain kinase and subsequent phosphorylation of myosin light chain-2 (Somlyo and Somlyo, 1994). In smooth muscle cells, Rho-kinase in concert with myosin light chain kinase acts to increase myosin light chain-2 phosphorylation (Shimokawa et al., 1999). However, in cardiac myocytes, the Rho-kinase inhibitor Y-27632 suppressed the endothelin-1-induced increase in Ca^{2+} transients with lower inhibition of cell shortening (Chu et al., 2002), in strong contrast to the inhibitory action of wortmannin in smooth muscle cells.

Myosin light chain-2 is also phosphorylated by myosin light chain kinase in the heart (Morano, 1999). Myosin light chain-2 may play an important role in the positive inotropic effects of an α_1-adrenergic receptor agonist (Thorburn and Thorburn, 1994) and endothelin-1 (Clement et al., 1992; Rossmanith et al., 1997; Morano, 1999). Recent findings suggest that phosphorylation of myosin light chain-2 via signaling processes triggered by activation of endothelin, angiotensin II, and α_1-adrenergic receptors plays a crucial role in myofilament Ca^{2+} sensitization in cardiac myocytes (Rossmanith et al., 1997; Andersen et al., 2002). Further support for the crucial role of myosin light chain-2 phosphorylation has been obtained by studying transgenic mice overexpressing nonphosphorylatable myosin light chain-2, since there was no shift in the Ca^{2+} sensitivity after treatment with myosin light chain kinase in transgenic mice, in contrast to the increase in Ca^{2+} sensitivity in nontransgenic mice (Sanbe et al., 1999). Rossmanith et al. (1997) reported that endothelin caused an increase in $[^{32}P]$ incorporation into myosin light chain-2, which suggests that the positive inotropic effect of endothelin may result from increased Ca^{2+} sensitivity caused by elevated myosin light chain-2 phosphorylation in cardiac muscle.

Protein kinase C activation triggered by stimulation with endothelin, angiotensin II, and α_1-adrenergic receptors may play a crucial role, since myosin light chain-2 phosphorylation by protein kinase C is associated with increases in the ATPase activity and Ca^{2+} sensitivity of force production (Clement et al., 1992; Noland et al., 1995). However, the role of protein kinase C activation is complex, since transgenic mice overexpressing different isoforms of protein kinase C show variable effects on the myofilament Ca^{2+} sensitivity, in that the sensitivity is decreased by protein kinase Cβ (Takeishi et al., 1998) and increased by protein kinase Cε (Takeishi et al., 2000).

4.2. Influence of wortmannin on isoproterenol-induced positive inotropic effect

Wortmannin had no effect on the isoproterenol-induced positive inotropic effect and an increase in Ca^{2+} transients, which suggests that the positive inotropic effect of isoproterenol is not dependent on myosin light chain kinase signal pathway (Andersen et al., 2002). Consistent with this view, the positive inotropic effect of β-adrenergic receptor

agonists was not associated with an increase in myosin light chain-2 phosphorylation in the heart (High and Stull, 1980, Davies et al., 2000).

4.3. Influence of wortmannin on basaline contractility

Wortmannin induced a negative inotropic effect but had no effect on the intracellular Ca^{2+} transients in rabbit cardiac myocytes. These findings suggest that the negative inotropic effect may be due to a decrease in Ca^{2+} sensitivity and that myosin light chain kinase activity may play an essential role in the maintenance of baseline contractility, as has been postulated in smooth muscle cells (Itoh et al., 1989). In various types of smooth muscle cells, wortmannin inhibits contraction without changing $[Ca^{2+}]_i$ (Burdyga and Wray, 1998; Longbottom et al., 2000; Ito et al., 2004). However, the role of myosin light chain kinase in the regulation of Ca^{2+} signaling may be largely dependent on the cell type, since the inhibition of myosin light chain kinase by wortmannin and a myosin light chain kinase antisense probe in endothelial cells blocked both thapsigargin- and agonist-induced Ca^{2+} mobilization (Watanabe et al., 2000). Although wortmannin was used in the present study, further studies using another inhibitor, such as ML-9 that has been shown to specifically inhibit the enzyme (Saitoh et al., 1987; Herring et al., 2000; Ito et al., 2004), are required to clarify the functional role of myosin light chain kinase in cardiac myocytes.

In summary, the current findings imply that the attenuated response to endothelin-1 induced by wortmannin may result from the inability of endothelin-1 to increase the myofilament Ca^{2+} sensitivity. Inhibition of myosin light chain kinase may reveal a differential role for the pathway in the contractile regulation induced by endothelin-1 and β-adrenergic receptor agonists in the rabbit ventricular myocardium.

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References

Andersen, G.G.O., Qvigstad, E., Schiander, I., Aass, H., Osnes, J.B., Skomedal, T., 2002. α_1-AR-induced positive inotropic response in heart is dependent on myosin light chain phosphorylation. Am. J. Physiol. 283, H1471–H1480.

Burdyga, T.V., Wray, S., 1998. The effects of inhibition of myosin light chain kinase by wortmannin on intracellular $[Ca^{2+}]$, electrical activity and force in phasic smooth muscle. Pflügers Arch.-Eur. J. Physiol. 436, 801–803.

Chu, L., Tanaka, H., Zhang, J.X., Norota, I., Ishii, K., Endoh, M., 2002. Involvement of Rho-kinase in regulation of contractility and cytosolic Ca^{2+} induced by crosstalk of endothelin-1 and norepinephrine in canine myocardium. Jpn. J. Pharmacol. 88 (suppl. 1), 64.

Chu, L., Takahashi, R., Norota, I., Miyamoto, T., Takeishi, Y., Ishii, K., Kubota, I., Endoh, M., 2003. Signal transduction and Ca^{2+} signaling in contractile regulation induced by crosstalk between endothelin-1 and norepinephrine in dog ventricular myocardium. Circ. Res. 92, 1024–1032.

Clement, O., Puceat, M., Walsh, M.P., Vassort, G., 1992. Protein kinase C enhances myosin light-chain kinase effects on force development and ATPase activity in rat single skinned cardiac cells. Biochem. J. 285, 311–317.

Davies, S.P., Reddy, H., Caivano, M., Cohen, P., 2000. Specificity and mechanism of action of some commonly used protein kinase inhibitors. Biochem. J. 351, 95–105.

Endoh, M., 1997. The role of phosphoinositide hydrolysis in the receptor-mediated inotropic response of the heart. Asia Pac. J. Pharm. 12, S11–S13 (suppl).

Endoh, M., Blinks, J.R., 1988. Actions of sympathomimetic amines on the Ca^{2+} transients and contractions of rabbit myocardium: reciprocal changes in myofibrillar responsiveness to Ca^{2+} mediated through α- and β-adrenoceptors. Circ. Res. 62, 247–265.

Endoh, M., Fujita, S., Yang, H.T., Talukder, M.A., Maruya, J., Norota, I., 1998. Endothelin: receptor subtypes, signal transduction, regulation of Ca^{2+} transients and contractility in rabbit ventricular myocardium. Life Sci. 62, 1485–1489.

Fujita, S., Endoh, M., 1996. 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. 2, S45–S57 (suppl).

Grynkiewicz, G., Poenie, M., Tsien, R.Y., 1985. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. J. Biol. Chem. 260, 3440–3450.

Herring, B.P., Dixon, S., Gallagher, P.J., 2000. Smooth muscle myosin light chain kinase expression in cardiac and skeletal muscle. Am. J. Physiol. 279, C1656–C1664.

High, C.W., Stull, J.T., 1980. Phosphorylation of myosin in perfused rabbit and rat hearts. Am. J. Physiol. 239, H756–H764.

Itoh, T., Ikebe, M., Kargacin, G.J., Hartshorne, D.J., Kemp, B.E., Fay, F.S., 1989. Effects of modulators of myosin light-chain kinase activity in single smooth muscle cells. Nature 338, 164–167.

Ito, S., Kume, H., Honjo, H., Kodama, I., Katoh, H., Hayashi, H., Shimokata, K., 2004. ML-9, a myosin light chain kinase inhibitor, reduces intracellular Ca^{2+} concentration in guinea pig trachealis. Eur. J. Pharmacol. 486, 325–333.

Longbottom, E.R., Lukas, M.J.M., Kupittayanant, S., Badrick, E., Shimigol, T., Wray, S., 2000. The effects of inhibiting myosin light chain kinase on contraction and calcium signaling in human and rat myometrium. Pflügers Arch.-Eur. J. Physiol. 440, 315–321.

Morano, I., 1999. Tuning the human heart molecular motors by myosin light chains. J. Mol. Med. 77, 544–555.

Noland Jr., T.A., Gou, X., Raynor, R.L., Jideama, N.M., Averyhart-Fullard, V., Solaro, R.J., Kuo, J.F., 1995. 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. 270, 25445–25454.

Qiu, Z., Wang, J., Perreault, C.L., Meuse, A.J., Grossman, W., Morgan, J.P., 1992. Effects of endothelin on intracellular Ca^{2+} and contractility in single ventricular myocytes from the ferret and human. Eur. J. Pharmacol. 214, 293–296.

Rossmanith, G.H., Hoh, J.F., Turnbull, L., Ludowyke, R.I., 1997. Mechanism of action of endothelin in rat cardiac muscle: cross-bridge kinetics and myosin light chain phosphorylation. J. Physiol. 505, 217–227.

Saitoh, M., Ishikawa, T., Matsushima, S., Naka, M., Hidaka, H., 1987. Selective inhibition of catalytic activity of smooth muscle myosin light chain kinase. J. Biol. Chem. 262, 7796–7801.

Sanbe, A., Fewell, J.G., Gulick, J., Osinska, H., Lorenz, J., Greg, H.D., Murray, L.A., Kimball, T.R., Witt, S.A., Robbins, J., 1999. Abnormal cardiac structure and function in mice expressing nonphosphorylatable cardiac regulatory myosin light chain 2. J. Biol. Chem. 274, 21085–21094.

Sato, S., Talukder, M.A.H., Sugawara, H., Sawada, H., Endoh, M., 1998. Effects of levosimendan on myocardial contractility and Ca^{2+} transients in aequorin-loaded right-ventricular papillary muscles and indo-1-loaded single ventricular cardiomyocytes of the rabbit. J. Mol. Cell. Cardiol. 30, 115–1128.

Shimokawa, H., Seto, M., Katsumata, N., Amano, M., Kozai, T., Yamawaki, T., Kuwata, K., Kandabashi, T., Egashira, K., Ikegaki, I., Asano, T., Kaibuchi, K., Takeshita, A., 1999. Rho-kinase-mediated pathway induces enhanced myosin light chain phosphorylations in a swine model of coronary artery spasm. Cardiovasc. Res. 43, 1029–1039.

Somlyo, A.P., Somlyo, A.V., 1994. Signal transduction and regulation in smooth muscle. Nature 372, 231–236.

Takanashi, M., Endoh, M., 1991. Characterization of positive inotropic effect of endothelin on mammalian ventricular myocardium. Am. J. Physiol. 261, H611–H619.

Takeishi, Y., Chu, G., Kirkpatrick, D.M., Li, Z., Wakasaki, H., Kranias, E.G., King, G.L., Walsh, R.A., 1998. In vivo phosphorylation of cardiac troponin I by protein kinase Cβ2 decreases cardiomyocyte calcium responsiveness and contractility in transgenic mouse hearts. J. Clin. Invest. 102, 72–78.

Takeishi, Y., Ping, P., Bolli, R., Kirkpatrick, D.L., Hoit, B.D., Walsh, R.A., 2000. Transgenic overexpression of constitutively active protein kinase Cε causes concentric cardiac hypertrophy. Circ. Res. 86, 1218–1223.

Talukder, M.A., Endoh, M., 1997. 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. 355, 87–96.

Talukder, M.A., Norota, I., Sakurai, K., Endoh, M., 2001. Inotropic response of rabbit ventricular myocytes to endothelin-1: difference from isolated papillary muscles. Am. J. Physiol. 281, H596–H605.

Thorburn, J., Thorburn, A., 1994. The tyrosine kinase inhibitor, genistein, prevents α-adrenergic-induced cardiac muscle cell hypertrophy by inhibiting activation of the Ras-MAP kinase signaling pathway. Biochem. Biophys. Res. Commun. 202, 1586–1591.

Watanabe, H., Tran, Q.K., Takeuchi, K., Fukao, M., Liu, M.Y., Kanno, M., Hayashi, T., Iguchi, A., Seto, M., Ohashi, K., 2000. Myosin light-chain kinase regulates endothelial calcium entry and endothelium-dependent vasodilation. FASEB J. 15, 282–284.

Yanagisawa, M., Kusumoto, H., Kimura, S., Tomobe, Y., Kobayashi, M., Mitsui, Y., Yazaki, Y., Goto, K., Masaki, T., 1988. A novel potent vasoconstrictor peptide produced by vascular endothelial cells. Nature 332, 411–415.

Yang, H.T., Sakurai, K., Sugawara, H., Watanabe, T., Norota, I., Endoh, M., 1999. 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. 126, 1785–1795.

Yano, H., Nakanishi, S., Kimura, K., Hanai, N., Saitoh, Y., Fukui, Y., Nonomura, Y., Matsuda, Y., 1993. Inhibition of histamine secretion by wortmannin through the blockade of phosphatidylinositol 3-kinase in RBL-2H3 cells. J. Biol. Chem. 268, 25846–25856.

Yomogida, S., Maruya, J., Norota, I., Ishii, K., Endoh, M., 2004. Differential inhibition by TAK-044 of the inotropic effects of endothelin-1 and endothelin-3. Eur. J. Pharmacol. 492, 217–224.