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# Rho-kinase-mediated pathway induces enhanced myosin light chain phosphorylations in a swine model of coronary artery spasm

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**Abstract**

**Objective:** We recently demonstrated in our swine model of coronary artery spasm that enhanced myosin light chain (MLC) phosphorylations (both MLC mono- and diphosphorylations) play a central role in the pathogenesis of the spasm. However, the molecular mechanism for and the phosphorylation sites for the enhanced MLC phosphorylations were unknown. In the present study, we addressed these points using hydroxyfasudil, a novel inhibitor of protein kinases, which we found preferentially inhibits Rho-kinase. **Methods:** The specificity of the inhibitory effects of hydroxyfasudil on Rho-kinase, MLCK, MRCKβ and PKC were examined by kinase assay in vitro. The left porcine coronary artery was chronically treated with interleukin-1β (IL-1β, 2.5 μg). Two weeks after the operation, coronary artery vasomotion was examined both in vivo and in vitro. MLC phosphorylations were examined by Western blot analysis and the sites for the phosphorylations by anti-phosphorylated MLC antibodies that identified the monophosphorylation site as Ser19 and diphophorylation sites as Ser19/Thr18 of MLC. **Results:** Inhibitory effects of hydroxyfasudil was at least 100 times more potent for Rho-kinase as compared with other protein kinases tested. Intracoronary serotonin (10 μg/kg) caused coronary hyperconstriction at the IL-1β-treated site in vivo, which was dose-dependently inhibited by hydroxyfasudil ($p<0.01$). The coronary segment taken from the spastic site also showed hypercontractions to serotonin in vitro, which were again dose-dependently inhibited by hydroxyfasudil ($p<0.01$). Western blot analysis showed that MLC monophosphorylation was significantly greater in the spastic segment than in the control segment, while MLC diphosphorylation was noted only at the spastic segment ($p<0.01$). The sites for the mono- and diphosphorylated MLC were identified as the monophosphorylated site Ser19 and diphosphorylated sites Ser19/Thr18 of MLC, respectively. Both types of MLC phosphorylations at the spastic segment were markedly inhibited by hydroxyfasudil ($p<0.01$). **Conclusion:** These results indicate that hydroxyfasudil-sensitive Rho-kinase-mediated pathway appears to mediate the enhanced MLC phosphorylations (on Ser19 and Ser19/Thr18 residues) and plays a central role in the pathogenesis of coronary artery spasm. 

*Keywords:* Coronary vasospasm; Rho-kinase; Myosin light chain; Calcium

## 1. Introduction

Coronary artery spasm plays an important role in a wide variety of ischemic heart diseases [1]. In order to elucidate the mechanism(s) for coronary spasm, we have developed swine models of the spasm, in which we demonstrated the pathogenetic importance of coronary atherosclerosis in general [2] and of inflammatory changes of the coronary artery in particular [3,4]. The spasm induced in our swine models has many similarities to that observed in humans,

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indicating that our models are useful in elucidating the pathogenesis of the spasm in humans [2–4].

We have recently demonstrated in our swine models that a protein kinase C (PKC)-mediated pathway is substantially involved [5,6] while enhanced myosin light chain (MLC) phosphorylations (both mono- and diphosphorylations) play a central role in the pathogenesis of the spasm [7]. We also showed that fasudil, an inhibitor of both MLC kinase (MLCK) and PKC [8,9], inhibited the coronary spasm and the enhanced MLC phosphorylations in our swine model [7]. However, the detailed intracellular mechanism for and the phosphorylation sites for the enhanced MLC phosphorylations remained to be elucidated.

Studies in vitro demonstrated that a GTP-binding protein regulates the receptor-mediated sensitization of the MLC phosphorylation [10] and that small GTPase Rho is involved in the GTP-enhanced $Ca^{2+}$ sensitivity of the smooth muscle contraction [11–13]. Recent studies further demonstrated that Rho regulates MLC phosphorylation through its targets, Rho-kinase and the myosin binding subunit (MBS) of myosin phosphatase [14,15]. Activated Rho interacts with Rho-kinase and MBS of myosin phosphatase, to activate Rho-kinase and translocate MBS. The activated Rho-kinase subsequently phosphorylates MBS, thereby inactivating myosin phosphatase [14]. Rho-kinase itself may phosphorylate MLC at the same site that is phosphorylated by MLC kinase, and activate myosin ATPase [15]. Activated form of Rho-kinase enhances MLC phosphorylation [16] and induces smooth muscle contraction [17], stress fiber formation and neurite retraction [18]. Both pathways, inhibition of myosin phosphatase and direct phosphorylation of MLC, may be involved in the increase in the MLC phosphorylation [17].

As we discuss later, we recently found that hydroxyfasudil, an active metabolite of fasudil [19], preferentially inhibits Rho-kinase. Thus, the present study was designed to further elucidate the intracellular mechanism for coronary spasm using hydroxyfasudil and to identify the phosphorylation sites for the enhanced MLC phosphorylations in our swine model. Our findings clearly indicated that hydroxyfasudil-sensitive Rho-kinase-mediated pathway appears to mediate the enhanced MLC phosphorylations (on Ser19 and Ser19/Thr18) and plays a central role in the pathogenesis of coronary artery spasm.

## 2. Methods

This experiment was reviewed by the Committee on Ethics in Animal Experiments of the Kyushu University School of Medicine and was carried out according to the *Guidelines for Animal Experiments* of the Kyushu University School of Medicine and the Law (No. 105) and Notification (No. 6) of the Japanese Government.

### 2.1. Kinase assay

The inhibitory effects of hydroxyfasudil on kinase activities were examined by kinase assay. The kinase reaction for GST (glutathione S-transferase)-Rho-kinase-CAT (catalytic domain) or GST–MRCKβ (myotonic dystrophy kinase-related Cdc42-binding kinase beta)-CAT was carried out in 50 μl of kinase buffer (50 mM Tris/HCl at pH 7.5, 2 mM EGTA, 1 mM EDTA, 5 mM $MgCl_2$, 3 mM KCl) containing 100 μM [γ-$^{32}$P]ATP (1–20 GBq/mmol), recombinant kinase, and 2 μM MLC. The kinase reaction for MLCK was carried out in 50 μl of kinase buffer (30 mM Tris/HCl at pH 7.5, 0.1 mM $CaCl_2$, 1 mM $MgCl_2$, 30 mM KCl, 5 μg/ml calmodulin) containing 100 μM [γ-$^{32}$P]ATP (1–20 GBq/mmol), MLCK, and 2 μM MLC. After incubation for 10 min at 30°C, the reaction mixtures were boiled in SDS sample buffer and subjected to SDS–PAGE. The radiolabelled bands were visualized by an image analyzer (Fuji).

The kinase reaction for GST-Rho-kinase-CAT or GST–MRCKβ-CAT was carried out in 50 μl of kinase buffer (50 mM Tris/HCl at pH 7.5, 2 mM EGTA, 1 mM EDTA, 5 mM $MgCl_2$) containing 100 μM [γ-$^{32}$P]ATP (1–20 GBq/mmol), GST-Rho-kinase-CAT, and 40 μM rsk kinase S6 substrate. The kinase reaction for PKC (α and β) was carried out in 50 μl of kinase buffer (20 mM Tris/HCl at pH 7.5, 0.5 mM $CaCl_2$, 5 mM $MgCl_2$, 20 μg/ml phosphatidyl serine, 100 nM 12-*O*-tetradecanoylphorbol β-acetate) containing 100 μM [γ-$^{32}$P]ATP (1–20 GBq/mmol), PKC, and 40 μM PKC pseudo-substrate peptide. After incubation for 10 min at 30°C, the reaction mixtures were spotted onto a Whatman p81 paper and washed with 75 mM phosphoric acid three times. Incorporation of $^{32}$P into the substrates was assessed by scintillation counting.

The inhibitory effect of hydroxyfasudil was expressed as IC50 values and inhibition constant ($K_i$) values, as reported previously [8,9].

### 2.2. Animal preparation

Male Yorkshire pigs weighing 25 to 30 kg were sedated with ketamine hydrochloride (12.5 mg/kg IM) and anesthetized with sodium pentobarbital (25 mg/kg IV). The animals were then intubated and ventilated with room air; oxygen was supplemented via a positive-pressure respirator (Shinano). Under aseptic conditions, a left thoracotomy was performed, and the proximal segments of the left anterior descending and circumflex coronary arteries were carefully dissected. The dissected segments of the coronary arteries were gently wrapped with cotton mesh that had absorbed 0.05 ml of sepharose bead suspension with recombinant human IL-1β (2.5 μg) [3,4,6]. We previously confirmed that the treatment with control beads alone causes no significant arteriosclerotic changes or vasospastic responses of the porcine coronary artery [3,4,6].

### 2.3. Preparation of IL-1β beads

IL-1β beads were prepared as follows [3,4]. One gram of sepharose microbeads (45 to 165 μm in diameter) was added to 50 ml of 1 mmol/l HCl solution and resuspended in 20 ml $NaHCO_3$/NaCl solution with 1 mg IL-1β. The beads were allowed to bind with IL-1β at room temperature for 1 h and then at 4°C overnight. After centrifugation at 1200 rpm for 5 min, the supernatant was separated, and the concentration of the remaining IL-1β in the supernatant was measured by an ELISA [3,4]. The IL-1β-bound beads in the pellet were resuspended in 20 ml $NaHCO_3$/NaCl solution and centrifuged four times at 1200 rpm for 5 min. Then the IL-1β-bound beads were resuspended with Tris–HCl buffer solution for 1 h and finally washed and resuspended so that the concentration of IL-1β was 50 μg/ml. All preparations were performed under sterile conditions [3,4,6].

Since in our bead preparation most of the IL-1β molecules are bound inside the beads by a covalent bond at the amino residues of the proteins, 1.2% or less of the IL-1β molecules are actually bound to the surface of the beads and biologically active. Thus, when 2.5 μg of IL-1β bound to the beads is applied to the coronary artery, <30 ng of IL-1β is biologically active [3].

### 2.4. In vivo experiment

Two weeks after the operation, we performed a coronary arteriographic study in which the coronary artery vasomotion was examined in vivo.

The animals were again anesthetized and ventilated as described above, and selective coronary arteriography was performed. A preshaped Judkins catheter was inserted into the right or left femoral artery, and then coronary arteriography in a left anterior oblique view was performed under control conditions and after intracoronary nitroglycerin 10 μg/kg. ECGs (leads I, II, III, V1, and V6), along with the mean arterial pressure and heart rate, were recorded continuously during the experiments. Coronary arteriography was repeated 2 min after the intracoronary administration of serotonin (10 μg/kg). Then, intracoronary administration of hydroxyfasudil at three different doses (10, 30, and 100 μg/kg) was performed, and the coronary vasomotion to serotonin was again evaluated at each dose of hydroxyfasudil.

The cineangiograms were projected on a screen using a cineprojector (ELX-35CB; Nishimoto Sangyo, Osaka, Japan), and an end-diastolic frame was selected and printed [3–6]. The coronary luminal diameters were measured by computer-assisted quantitative coronary angiogram system (Sony, Tokyo, Japan). The degree of constrictive response was expressed as the percent decrease in the luminal diameter from the control level. The coronary diameter was measured at the segments treated with IL-1β as well as at the untreated segments of a comparable diameter [3–6].

### 2.5. In vitro experiment

Three to four days after the in vivo experiments, when the effects of hydroxyfasudil had totally disappeared, the animals were sedated with ketamine hydrochloride (12.5 mg/kg IM) and sacrificed with a lethal dose of sodium pentobarbital, exsanguinated, and then the heart was excised. The coronary arteries at the IL-1β-treated and control sites were carefully dissected and cleaned of any perivascular tissue and cut into rings measuring ~4 mm in length. The strips were fixed vertically between hooks in an organ bath of 20 ml capacity containing Krebs–Henseleit solution, which was maintained at 37°C and aerated with a mixture of 95% $O_2$−5% $CO_2$ [7]. The hook anchoring the upper end of the strip was connected to the lever of a force transducer (Nihon-Kohden Kogyo, Tokyo, Japan). The resting tension was adjusted to 5 g. KCl solution (62 mM) was applied every 15–20 min until the amplitude of the contraction reached a constant value. The tension was represented as a percentage of the tension attained in the last precontraction with 62 mM KCl. The contractions to serotonin were examined in the absence and presence of different doses of hydroxyfasudil ($10^{-6}$ and $10^{-5}$ mol/l), which was added 10 min before addition of serotonin.

### 2.6. Measurements of MLC phosphorylations

The extent of MLC phosphorylation in the strips was measured by separation of non-, mono-, and diphosphorylated forms by glycerol–polyacrylamide gel electrophoresis (PAGE) followed by electrophoretic transfer of the proteins to a nitrocellulose membrane. The relative amounts of each form were quantified by immunoblot procedures, as described previously [7].

Rings mounted for isometric studies were frozen by immersion in acetone containing 10% trichloroacetic acid (TCA) and 10 mM dithiothreitol (DTT) cooled with dry ice. Frozen tissues were washed twice with acetone containing 10 mM DTT to remove the TCA and then dried. The dried ring was cut into small pieces, exposed to 80 μl of glycerol–PAGE sample buffer for purposes of extraction, and then passed through a 0.45-μm membrane filter. The urea-solubilized samples (5 μl) were subjected to glycerol–PAGE/immunoblot analysis, using the specific MLC antibody [18]. The region containing MLC was visualized using an ECL Western blotting system (Amersham). The extent of MLC phosphorylation is expressed as the percent MLC in the mono- and diphosphorylated forms, respectively. The validity of the MLC phosphorylation assay system was demonstrated by using MLC specific phosphatase purified from chicken gizzard [20].

### 2.7. Measurement of phosphorylation on Ser19 and Ser19/Thr18 of MLC

Relative amounts of phosphorylated Ser19 residue or Ser19/Thr18 residues in the MLC molecule in arteries were determined using SDS PAGE followed by immunoblot method using specific antibodies which specifically recognize monophosphorylated MLC at Ser19 (anti-MLC-P antibody) [21] or diphosphorylated MLC at Ser19 and Thr18 (anti-MLC-P2 antibody) [22]. SDS sample buffer (15 μl) containing 12.5% SDS, 25% 2-mercaptoethanol, 50 mM tris(hydroxymethyl) aminomethane, 50% sucrose and 0.1% bromophenol blue was added to the above mentioned urea-solubilized samples (60 μl), which were then boiled for 5 min. The samples (20 μl each) were applied to three sets of 15% polyacrylamide SDS gels (BioRad, Richmond, CA) and subjected to electrophoresis at 20 mA for 3 h. Electophoretic transfer of proteins from SDS–PAGE gels onto nitrocellulose membranes were carried out in buffer containing 20 mM tris (hydroxymethyl) aminomethane, 20% methanol, with application of 6 V/cm for 1 h. Three sets of nitrocellulose membranes were incubated with anti-MLC antibody, anti-MLC-P antibody or anti-MLC-P2 antibody, respectively. For detection of immunoreacted bands, we used an ECL Western blotting system (Amersham). The ratio of staining density of Ser19 or Ser19/Thr18 to that of total MLC in preparations before stimulation with serotonin was expressed as 1.0.

### 2.8. Materials and chemicals

GST-Rho-kinase-CAT (6-553 amino acids) and GST–MRCKβ-CAT (1–550 amino acids) were produced in Sf9 cells with a baculovirus system [23] and purified on a glutathione–sepharose column [24]. rsk kinase S6 substrate (RRRLSSLRA) and PKC pseudo-substrate (RFARKGSLRQKNVHEVK) were synthesized. [γ-$^{32}$P]ATP was purchased from Amersham. Serotonin (5-hydroxytryptamine) was purchased from Sigma Chemical, and hydroxyfasudil [19] was provided from Asahi Chemical. Dilution was done with a physiological salt solution.

### 2.9. Statistical analysis

The results were expressed as mean±SEM. Throughout the text, *n* represents the number of animals tested. A repeated-measures ANOVA was performed to evaluate global statistical significance, and if a significant *F* value was found, Scheffe's test was performed to identify the difference among the groups. A value of $p<0.05$ was considered to be statistically significant.

## 3. Results

### 3.1. Specific inhibitory effects of hydroxyfasudil on Rho-kinase

When examined using MLC as a substrate, hydroxyfasudil potently inhibited the activity of recombinant Rho-kinase in a dose-dependent manner (IC50 1.8 μM), while its inhibitory effect was markedly (at least 100 times) less for MRCKβ or MLCK (Fig. 1A). When examined using peptide as a substrate, hydroxyfasudil again potently

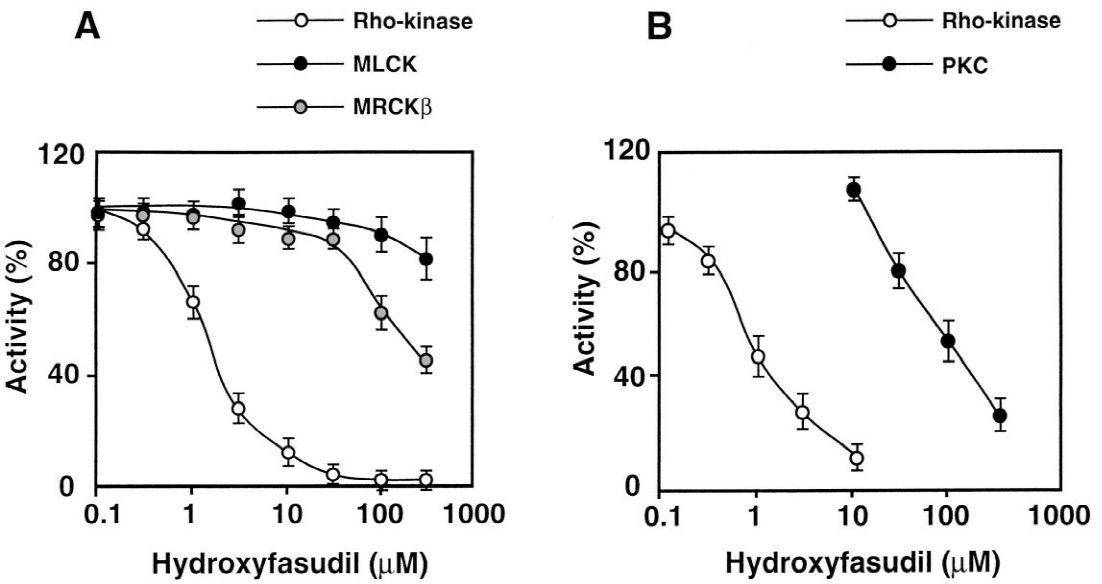


Fig. 1. Specific inhibition of Rho-kinase by hydroxyfasudil in vitro. A, Inhibitory effects of hydroxyfasudil when examined using MLC as a substrate; B, inhibitory effects of hydroxyfasudil when examined using a peptide as a substrate. MLCK, myosin light chain kinase; MRCKβ, the protein kinase with the kinase domain similar to that of Rho-kinase; PKC, protein kinase C.

inhibited Rho-kinase (IC50 0.9 μM), while its inhibitory effect was markedly less for PKC (IC50 100 μM) (Fig. 1B). These results indicate that hydroxyfasudil preferentially inhibits the Rho-kinase activity in vitro.

### 3.2. In vivo study

Two weeks after the operation, serotonin (10 μg/kg IC) repeatedly caused hyperconstriction at the IL-1β-treated site in vivo (Fig. 2). The pretreatment with hydroxyfasudil did not significantly change heart rate or blood pressure (data not shown). This pretreatment with hydroxyfasudil dose-dependently inhibited the serotonin-induced coronary spasm at the IL-1β-treated site in vivo, while at the control site its inhibitory effect on the serotonin-induced contraction was not evident (Figs. 2 and 3).

### 3.3. In vitro study

In organ chamber experiments, serotonin (1 μmol/l) induced a contraction of the IL-1β-treated and control coronary segments with endothelium, which rapidly developed and reached a maximum after the first 5–8 min, followed by a slight decrease and then by a sustained response. Serotonin caused hypercontractions in the IL-1β-treated coronary segments compared to the control segments in vitro (Fig. 4). The pretreatment with hydroxyfasudil dose-dependently inhibited the serotonin-induced

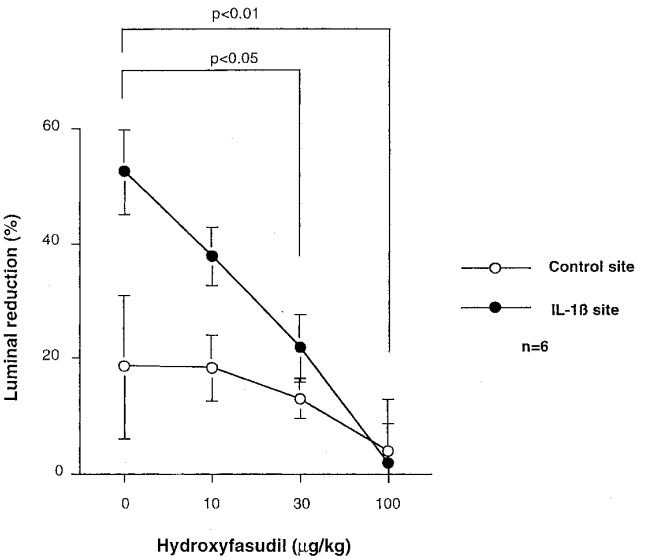


Fig. 3. Inhibitory effect of hydroxyfasudil on the serotonin (10 μg/kg IC)-induced coronary hyperconstriction in vivo. Data are presented as mean±SEM.

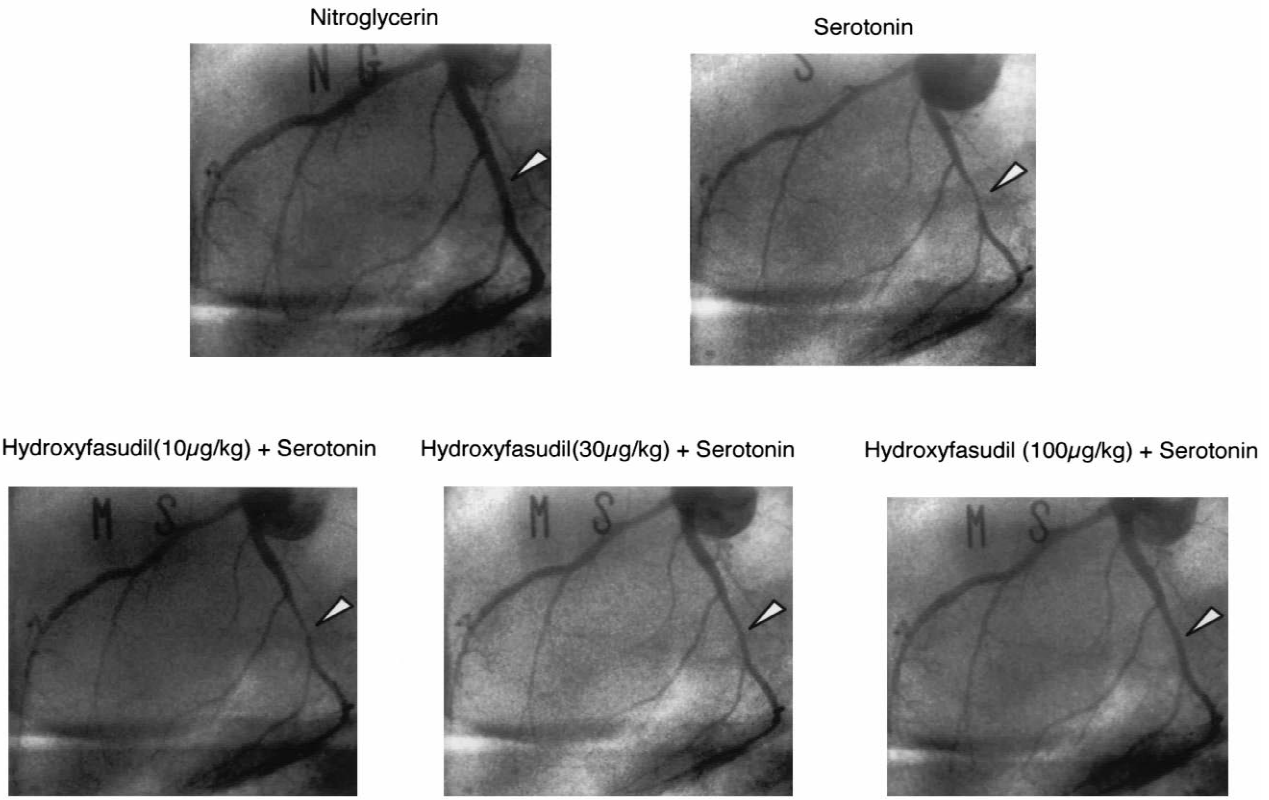


Fig. 2. Coronary angiograms 2 weeks after chronic treatment with IL-1β. After intracoronary nitroglycerin (10 μg/kg), mild stenotic lesion was noted at the IL-1β-treated site (arrow) (left upper), where intracoronary serotonin (10 μg/kg) repeatedly induced coronary hyperconstriction (right upper). This serotonin-induced coronary hyperconstriction was dose-dependently inhibited by pretreatment with intracoronary hydroxyfasudil (10, 30, and 100 μg/kg) (lower three panels).

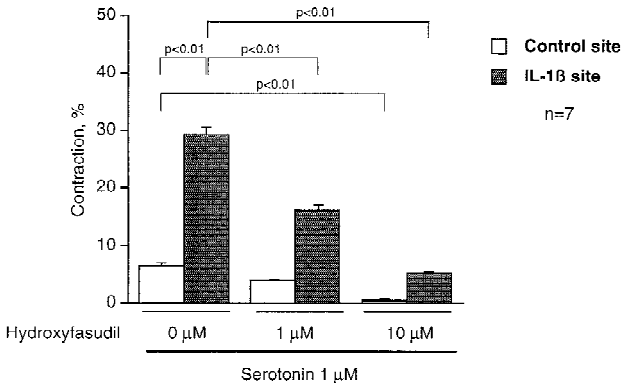


Fig. 4. Inhibitory effect of hydroxyfasudil on the serotonin (1 μmol/l)-induced contraction of isolated coronary rings with endothelium in vitro. The extent of contraction is expressed as percentage of the contraction to 62 mmol/l KCl. Data are presented as mean±SEM.

hypercontractions of the spastic coronary segments, while higher concentrations of hydroxyfasudil also inhibited the serotonin-induced contractions of the normal coronary segments (Fig. 4).

### *3.4. MLC phosphorylations*

The extent of MLC mono- and diphosphorylation was measured when the serotonin-induced contraction of each ring reached a maximum. Glycerol–PAGE followed by Western blot analysis showed that MLC monophosphorylation was significantly increased both in the IL-1β-treated and the control segments (Figs. 5 and 6), while MLC diphosphorylation was noted only at the IL-1β-treated segment (Figs. 5 and 6). In the spastic coronary segments, the enhanced MLC monophosphorylations were markedly and dose-dependently inhibited by hydroxyfasudil to the levels under control conditions, while the MLC diphosphorylations were abolished by hydroxyfasudil (Fig. 6). In contrast, in the control coronary segments hydroxyfasudil inhibited the increased MLC monophosphorylations to the levels under control conditions (Fig. 6).

The increase in MLC monophosphorylations tended to be correlated with and that in MLC diphosphorylations significantly correlated with the extent of the serotonin-induced contractions (Fig. 7). When the increases in MLC mono- and diphosphorylations were combined, the summed increase in MLC phosphorylations significantly correlated with the extent of serotonin-induced contractions (Fig. 7).

### *3.5. Measurement of phosphorylation on Ser19 and Ser19/Thr18 of MLC*

Relative amount of phosphorylated Ser19 residue of the MLC molecule in the IL-1β-treated and control arteries were compared with that of total monophosphorylated MLC determined using glycerol–PAGE followed by the immunoblot method (Fig. 8A). There was a close analogy between the relative amount of phosphorylated Ser19 in MLC and total monophosphorylated MLC. These results suggest that the phosphorylation site of monophosphorylated form of MLC induced by serotonin is mainly Ser 19 residue of MLC in both the spastic and control arteries.

Relative amount of phosphorylated Ser19/Thr18 residues on the MLC molecule increased in IL-1β-treated arteries (Fig. 8B). However, we were unable to compare the relative amount of phosphorylated Ser19/Thr18 with total diphosphorylated MLC determined using glycerol-PAGE followed by immunoblot method, because the

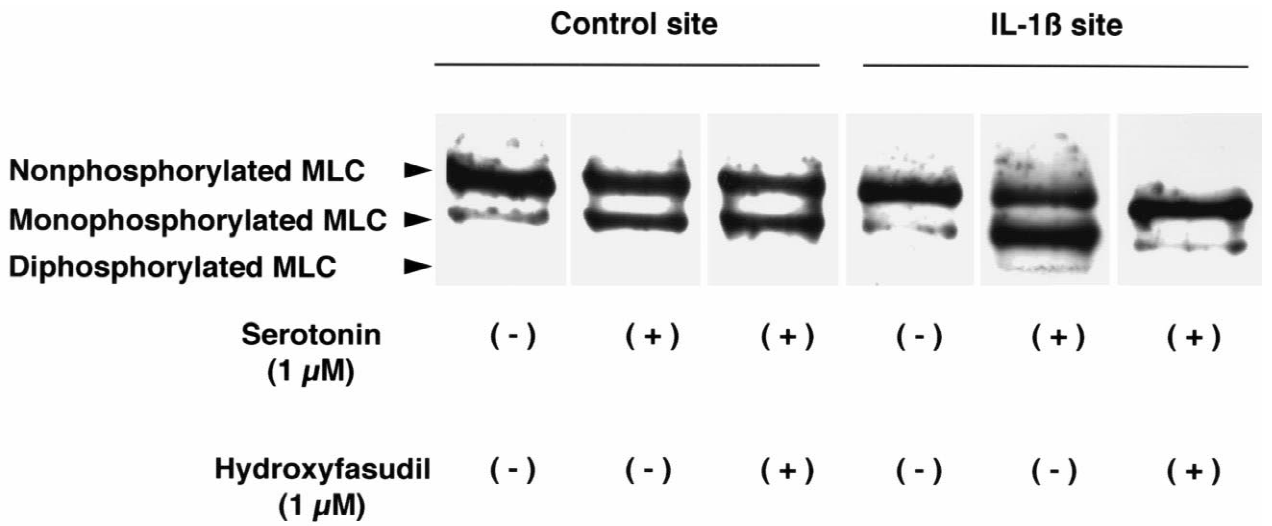


Fig. 5. Immunoblotting for MLC of the porcine coronary artery with and without serotonin (1 μmol/l). MLC monophosphorylation was increased in response to serotonin in both IL-1β-treated and control segments, while MLC diphosphorylation was noted only in the IL-1β-treated segment. Hydroxyfasudil exerted an inhibitory effect on both MLC mono- and diphosphorylations.

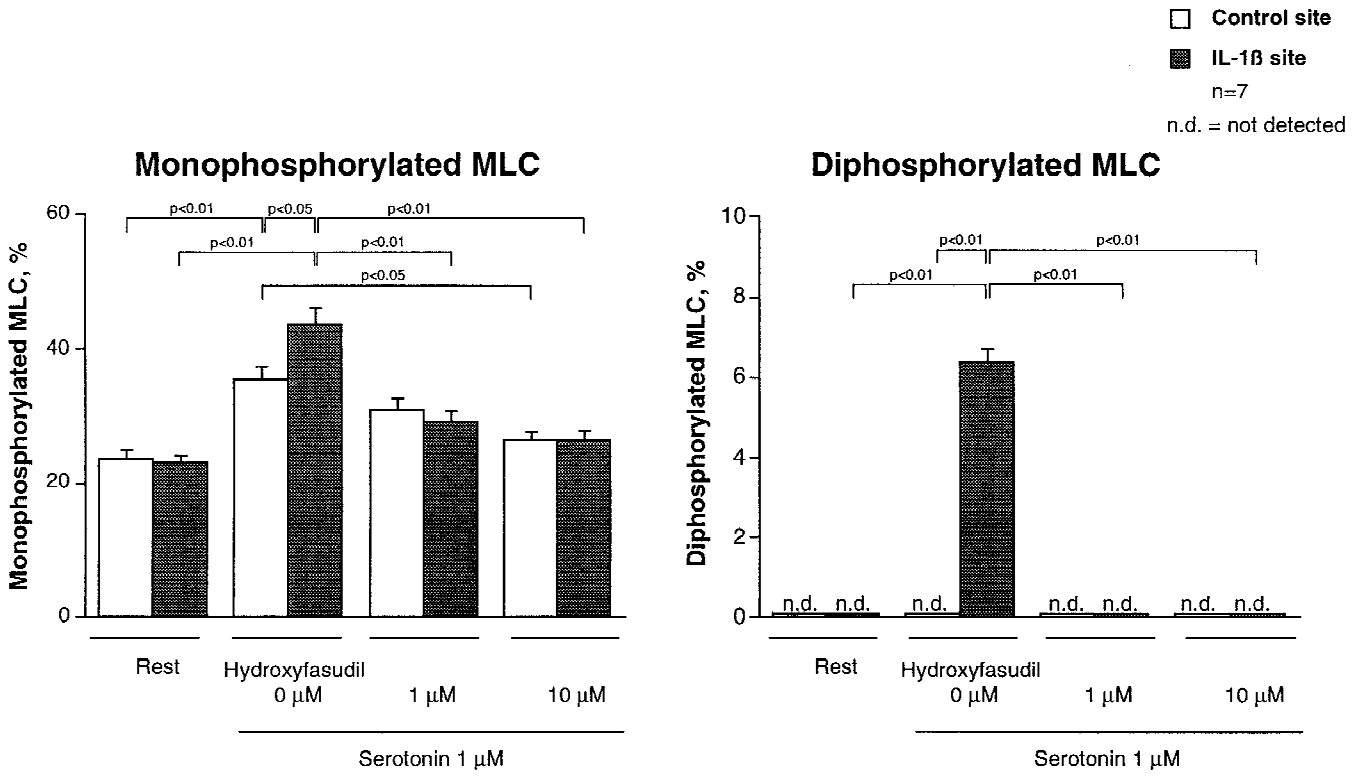


Fig. 6. MLC monophosphorylation (left) and MLC diphosphorylation (right) of the coronary artery (with endothelium) under control conditions and in response to serotonin (1 μmol/l). Serotonin-induced MLC monophosphorylation was significantly enhanced in the control and IL-1β-treated segments and was dose-dependently inhibited by hydroxyfasudil. Serotonin-induced MLC diphosphorylation was noted only in the IL-1β-treated segment and was abolished by hydroxyfasudil. n.d.=not detected. Data are presented as mean±SEM.

diphosphorylated MLC in non-stimulated arteries could not be detected by glycerol-PAGE method. The anti-MLC-P2 antibody may be much more sensitive in recognizing the phosphorylated Ser19/Thr18 residues on the MLC as compared with anti-MLC antibody.

## 4. Discussion

The novel findings of the present study were that (a) hydroxyfasudil is a specific inhibitor of Rho-kinase, (b) the mono- and diphosphorylation sites of MLC in the spastic

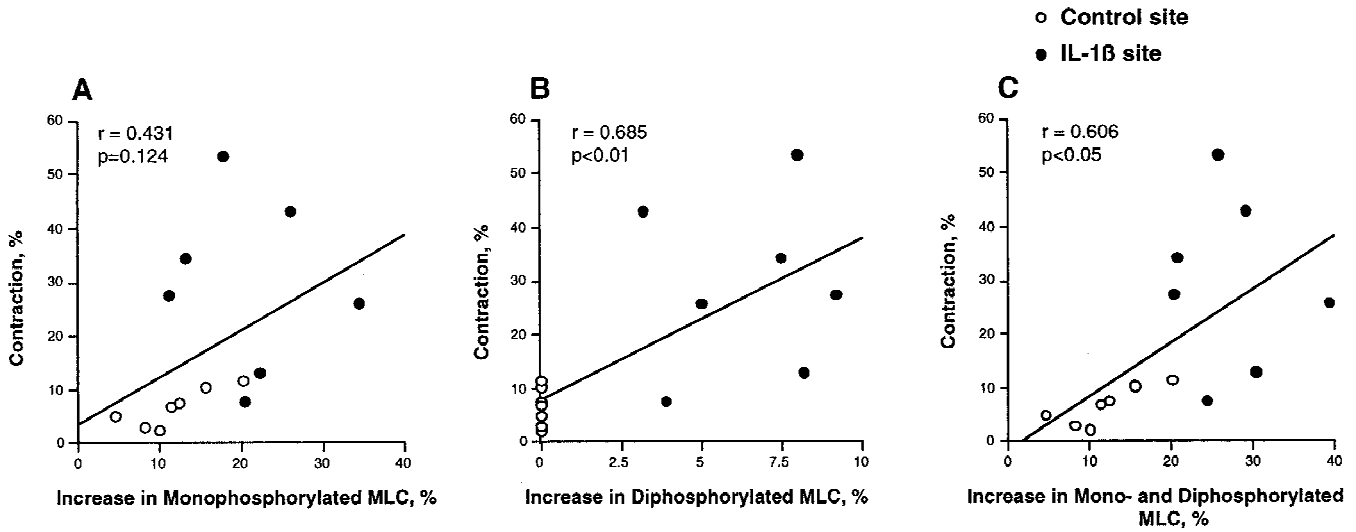


Fig. 7. Correlation between serotonin-induced contractions and the increase in MLC monophosphorylations (A), between those contractions and the increase in MLC diphosphorylations (B), and between those contractions and the increase in summed MLC phosphorylations (C).

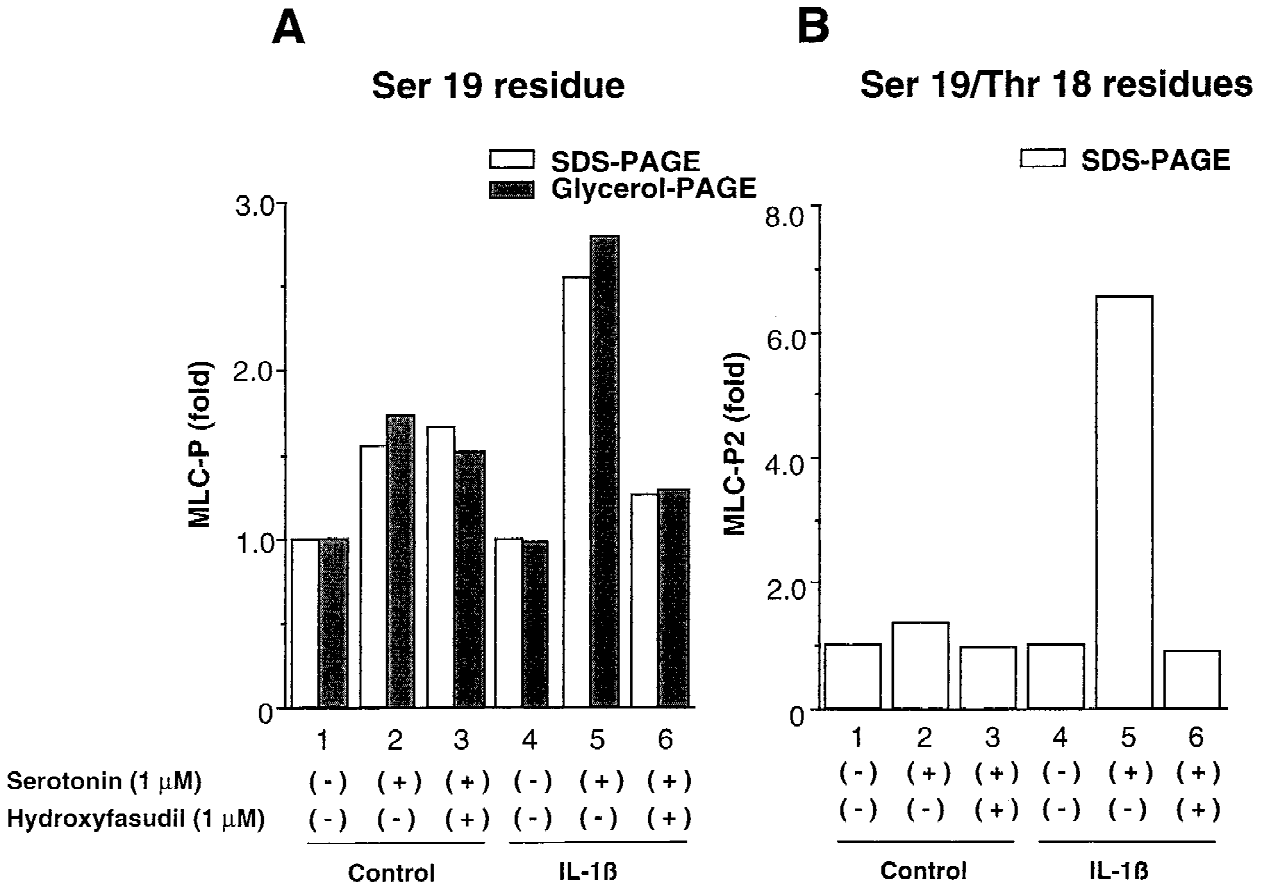


Fig. 8. Measurement of phosphorylated Ser19 residue and Ser19/Thr18 residues of the MLC molecule in the IL-1β-treated and control arteries. Relative amount of phosphorylated Ser19 residue (A) or Ser19/Thr18 residues (B) in the MLC molecule in arteries were determined using SDS PAGE followed by the immunoblot method. Relative amount of monophosphorylated MLC (A) was determined by glycerol-PAGE followed by immunoblot method. The level of MLC phosphorylation before stimulation with serotonin is expressed as 1.0. Each value is the mean of two experiments.

artery were Ser19 and Ser19/Thr18, respectively and (c) hydroxyfasudil significantly inhibited the enhanced MLC phosphorylations as well as the spasm both in vivo and in vitro. Thus, the present study clearly demonstrates that the enhanced MLC phosphorylations in the vascular smooth muscle play a central role in the pathogenesis of coronary spasm and that hydroxyfasudil-sensitive Rho-kinase-mediated pathway appears to be involved in those enhanced MLC phosphorylations. To the best of our knowledge, this is the first report that elucidated the important role of Rho-kinase-mediated pathway in the pathogenesis of coronary artery spasm and identified the MLC phosphorylation sites of the spastic coronary artery.

### *4.1. Phosphorylation sites of MLC in the spastic coronary artery*

We have recently demonstrated that coronary spasm is associated with an enhanced and sustained MLC monophosphorylation and an appearance of MLC diphosphorylation [7] (Fig. 9). It has been reported that MLC is phosphorylated by MLCK and PKC in vitro [25,26]. Recently Rho-kinase has also been reported to phosphorylate MLC in vitro [15]. MLCK and Rho-kinase preferentially phosphorylates Ser19 residues on MLC and activates myosin ATPase [25,27,28]. Phosphorylation of the second site (Thr18) of MLC by MLCK further increased myosin ATPase activity [29]. PKC also phosphorylates MLC at multiple sites (Ser1, Ser2 and Thr9), however, PKC can not activate myosin ATPase [26]. Thus, it is important to determine the phosphorylated sites of MLC in the spastic artery. Glycerol–PAGE followed by the immunoblot method using anti-MLC antibody does not quantitatively separate the MLCK or Rho-kinase-dependent phosphorylation of MLC from PKC-dependent one. We raised a monoclonal antibody against a Ser19 site phosphorylated MLC [21] and a polyclonal antibody against Ser19/Thr18 sites phosphorylated MLC [22]. Using these antibodies, we were able to demonstrate that phosphorylations of Ser19 site and Ser19/Thr18 sites of MLC were enhanced in the spastic coronary arteries. We did not examine the phosphorylations of Ser1, Ser2, or Thr9 residues, which are directly mediated by PKC [26]. However, the contribution of these MLC phosphorylations

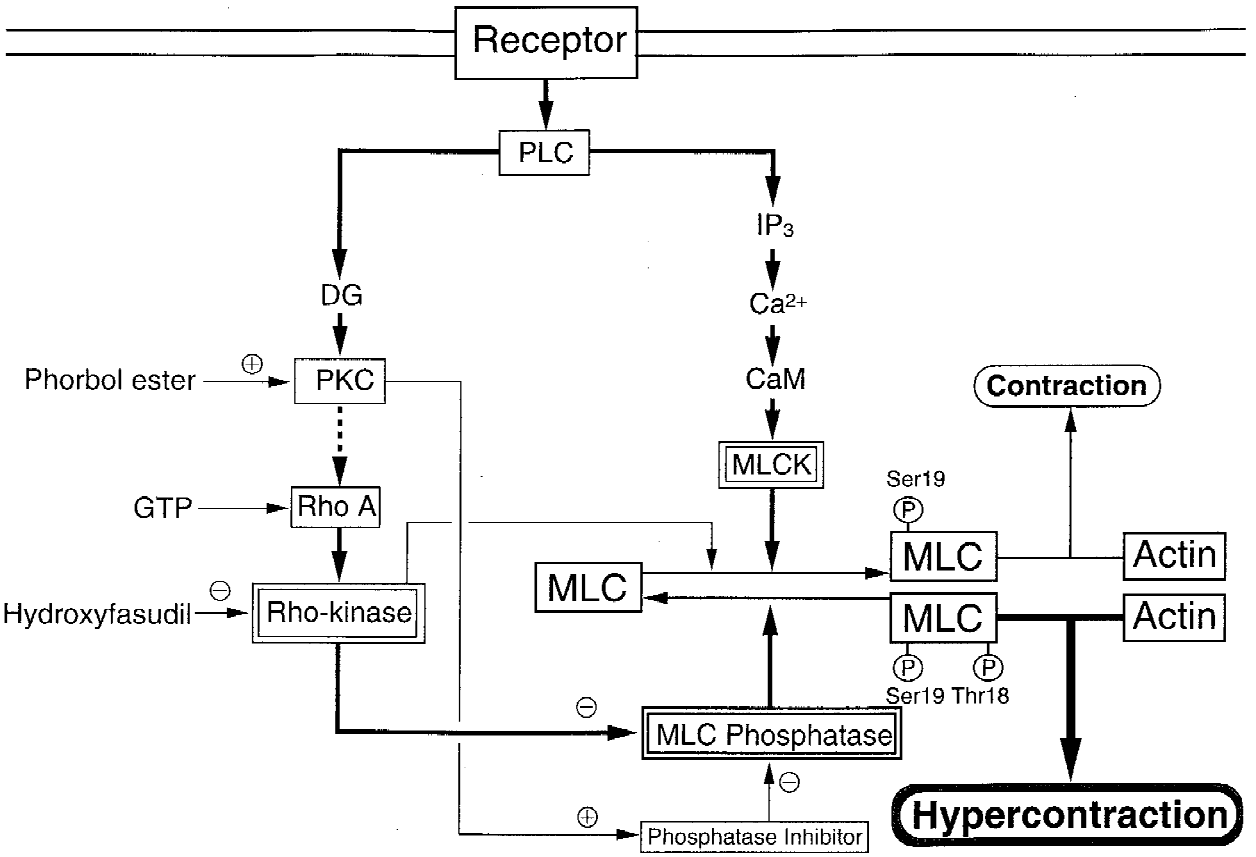


Fig. 9. Working hypothesis of the intracellular mechanisms for coronary artery spasm. PLC=phospholipase C, DG=diacylglycerol, PKC=protein kinase C, GTP=guanosine triphosphate, IP3=inositol 1,4,5-triphosphate, CaM=calmodulin, MLC=myosin light chain, Ser19=Ser19 residue, Thr18=Thr18 residue, (+)=stimulation, (−)=inhibition. For the occurrence of the spasm, the Rho-kinase-mediated pathway may play an important role, while the contribution of intracellular $Ca^{2+}$ release may be minimal. Regarding the Rho-kinase-mediated pathway, several alterations may be involved, including an enhanced expression of Rho-kinase, an increased Rho-kinase activity, and an inhibition of myosin phosphatase activity, all of which could eventually enhance the MLC phosphorylations.

to the occurrence of the spasm may be minimal partly because the phosphorylations of Ser19 site and Ser19/Thr18 sites of MLC are largely responsible for the spasm (e.g. positive correlation between the phosphorylations and the developed tensions) and partly because PKC does not activate myosin ATPase or does not lead force generation or contractions [24].

### *4.2. Rho-kinase-mediated pathway and coronary spasm*

We found that hydroxyfasudil preferentially inhibits Rho-kinase compared to MLCK, MRCK β, or PKC (Fig. 1). In the present study, hydroxyfasudil preferentially inhibited the enhanced components of coronary artery contractions and the MLC phosphorylations at the spastic site, while at the control site its inhibitory effects on the contractions and MLC phosphorylations were less prominent. Hydroxyfasudil is quite different from fasudil as it does not inhibit MLCK; calculated $K_i$ value of hydroxyfasudil for MLCK is more than 300 μM (Fig. 1), which is in contrast to the previously reported $K_i$ value of fasudil for MLCK (36 μM). These results suggest that hydroxyfasudil-sensitive Rho-kinase-mediated pathway plays an important role in the enhanced MLCK-dependent MLC phosphorylations in the spastic coronary artery (Fig. 9).

The level of MLC phosphorylation is determined by a balance between MLC phosphorylation and dephosphorylation [25,27] (Fig. 9). We recently suggested that the generation of diphosphorylated MLC may be caused in part by the inhibition of MLC phosphatase in smooth muscle cells [30]. We also showed that treatment with 10–100 nmol/l calyculin A, a protein phosphatase inhibitor, potently induced MLC diphosphorylation in smooth muscle cells without an increase in intracellular $Ca^{2+}$ levels [30]. Recently we found that the direct increase in intracellular $Ca^{2+}$ levels by the $Ca^{2+}$ ionophore did not result in an increase in diphosphorylated MLC (unpublished data). Noda et al. [12] reported that in permeabilized porcine aortic smooth muscle cells the increase in intracellular $Ca^{2+}$ levels caused monophosphorylation of MLC alone, while additional treatment with GTP-γS, which is thought to inactivate MLC phosphatase, caused both

mono- and diphosphorylation of MLC. These results suggest that inhibition of MLC phosphatase activity is essential for the induction of MLC diphosphorylation in vascular smooth muscle cells. We consider that the regulatory mechanism of MLC phosphatase activity may be altered in the spastic coronary artery, where the resultant inactivation of MLC phosphatase may cause both the enhanced MLC monophosphorylation and the appearance of MLC diphosphorylation, resulting in the occurrence of coronary artery spasm (Fig. 9). Indeed, we have recently demonstrated that the mRNA expression of Rho-kinase is markedly upregulated at the spastic coronary segment in the present model of coronary artery spasm with IL-1β (unpublished observations).

Recent studies showed that smooth muscle myosin phosphatase consists of 38-kDa catalytic subunit, the 130 kDa myosin binding subunit (MBS) and the 21 kDa subunit [31–33]. MBS serves as a targeting subunit of myosin phosphatase to myosin and enhances the activity of the enzyme toward myosin [31]. We recently reported that Rho-kinase phosphorylated the MBS and reduced the myosin phosphatase activity in vitro [14]. The induced expression of an activated mutant of Rho in NIH3T3 fibroblasts increased the extent of MLC phosphorylation with an increase in the phosphorylation level of MBS, suggesting that the phosphorylation of MBS is involved in Rho-mediated regulation of MLC phosphorylation [14]. In the present study, hydroxyfasudil, which selectively inhibits Rho-kinase, preferentially inhibited the enhanced components of the MLC phosphorylations at the spastic site. Thus, it is highly possible that Rho-kinase-mediated suppression of myosin phosphatase activity is selectively enhanced in the spastic arteries. Myosin phosphatase activity and the MBS phosphorylation level in the spastic coronary artery remains to be determined in a future study. A novel MLC phosphatase inhibitor that is potentiated by PKC has recently been isolated from porcine aorta media [34], and the possible involvement of this endogenous MLC phosphatase inhibitor in our model also remains to be examined.

Recently it has been shown that Y27632, another inhibitor of Rho-kinase, inhibits smooth muscle contraction by inhibiting $Ca^{2+}$ sensitization and decreases blood pressure in several rat models of hypertension but not in normotensive animals [35]. Thus, Rho-kinase-mediated $Ca^{2+}$ sensitization of smooth muscle contraction may also be involved in the pathophysiology of certain types of hypertension.

In summary, we were able to demonstrate that hydroxyfasudil-sensitive Rho-kinase-mediated pathway mediate the enhanced MLC phosphorylations and coronary artery spasm. The molecular mechanism(s) for the enhanced involvement of the Rho-kinase-mediated pathway at the inflammatory/arteriosclerotic coronary segment should be examined in a future study.

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