Evidence for Protein Kinase C-Mediated Activation of Rho-Kinase in a Porcine Model of Coronary Artery Spasm

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Objective—We have recently demonstrated that protein kinase C (PKC) and Rho-kinase play important roles in coronary vasospasm in a porcine model. However, it remains to be examined whether there is an interaction between the two molecules to cause the spasm.

Methods and Results—A segment of left porcine coronary artery was chronically treated with IL-1β–bound microbeads in vivo. Two weeks after the operation, phorbol ester caused coronary spasm in vivo and coronary hypercontractions in vitro at the IL-1β–treated segment; both were significantly inhibited by hydroxyfasudil, a specific Rho-kinase inhibitor. Guanosine 5’-[γ-thio]triphosphate (GTPγS), which activates Rho with a resultant activation of Rho-kinase, enhanced Ca2+ sensitization of permeabilized vascular smooth muscle cells, which were resistant to the blockade of PKC by calphostin C. The GTPγS-induced Ca2+ sensitization was greater in the spastic segment than in the control segment. Western blot analysis revealed that only PKCδ isoform was activated during the hypercontraction.

Conclusions—These results demonstrate that PKC and Rho-kinase coexist on the same intracellular signaling pathway, with PKC located upstream on Rho-kinase, and that among the PKC isoforms, only PKCδ may be involved. Thus, the strategy to inhibit Rho-kinase rather than PKC may be a more specific and useful treatment for coronary spasm.

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Key Words: arteriosclerosis • coronary disease • smooth muscle • signal transduction

Coronary artery spasm plays an important role in the pathogenesis of a variety of ischemic heart diseases, including not only variant angina but also other forms of angina, myocardial infarction, ventricular arrhythmias, and sudden death.1,2 However, the intracellular mechanism for the spasm remains to be elucidated.

We previously developed a porcine model of coronary spasm in which the spasm was repeatedly induced by autacoids, such as serotonin and histamine, at atherosclerotic lesions made by a combination of balloon endothelial removal and high-cholesterol feeding.3-5 We subsequently developed a porcine model in which long-term adventitial treatment with interleukin-1β (IL-1β), one of the major inflammatory cytokines, induces arteriosclerotic changes and vasospastic responses of the coronary artery.6-15 Since the histological changes and vasospastic responses in our porcine models are similar to those observed in humans, our models may be useful to elucidate the mechanisms of the spasm in humans.

We recently demonstrated that in our porcine models, coronary artery spasm induced by serotonin is mediated by protein kinase C (PKC)5-7 and Rho-kinase.9,11-15 Rho-kinase, one of the major target proteins of small Rho GTPase,16,17 was upregulated in spastic coronary segment, leading to an increased phosphorylation of myosin-binding subunit of myosin phosphatase (MLCPh) and the resultant suppression of MLCPh.11-15 Indeed, we recently demonstrated that Rho-kinase is substantially involved in hypercontractions of isolated arteriosclerotic human arteries in vitro18 as well as in coronary artery spasm of patients with vasospastic angina in vivo.19 By contrast, we also demonstrated that phorbol-12,13-dibutyrate (PDBu), a direct activator of PKC, induced coronary spasm, which was significantly inhibited by pretreatment with PKC inhibitors.5,7 However, it remains to be elucidated whether there is an interaction between these two important signaling molecules and if so, how they interact in agonists-induced smooth muscle hypercontractions. Thus, the present study was designed to address these important questions.

Methods
This study was reviewed by the Ethics Committee on Animal Experiment at Kyushu University and was carried out in accordance with the...
Effect of Rho-Kinase Inhibitor and PKC Inhibitor on GTPγS-Induced Contractions of Permeabilized Coronary Smooth Muscle

For permeabilized preparations of porcine coronary arteries, the skinning method was used as previously described. Briefly, a small strip (0.1 mm in width and thickness and 0.6 mm in length) of vascular smooth muscle fibers was prepared from the blood vessel. Mechanical activity of the muscle fibers was measured by a strain gauge in a chamber with 0.8 mL capacity. The muscle fibers were stretched to an optimal length where KCl (62 mmol/L)-induced tension was maximal, and isometric contractions were recorded. After recording the KCl-induced contractions, the smooth muscle fibers were permeabilized with 50 μg/mL saponin dissolved in Ca²⁺-free relaxing solution for 15 minutes at 25°C. Two protocols were then performed. First, to examine the effect of hydroxyfasudil, a specific inhibitor of Rho-kinase, on GTP-mediated smooth muscle contractions, 10 μmol/L guanosine 5’-O-[3-Thio]triphosphate (GTPγS) was applied in the fixed submaximal concentration of Ca²⁺ (pCa = 7.0), followed by additional administration of 3 μmol/L hydroxyfasudil. Second, to examine the involvement of PKC, the same experiment was performed using 1 μmol/L calphostin C, a specific PKC inhibitor.

Western Blot Analysis for PDBu-Induced Membrane Translocation of RhoA

To clarify the PKC-mediated activation of the Rho/Rho-kinase signaling pathway, membrane translocation of RhoA was examined. Ring specimens of endothelium-removed porcine coronary arteries with or without treatment with PDBu (0.01 μmol/L) were immediately frozen in liquid nitrogen and were homogenized in lysis buffer. After the sample was centrifuged at 25 000g for 3 hours to generate membrane and cytosolic fractions, equal amounts of protein from both fractions were subjected to SDS-PAGE/immunoblot analysis using a specific RhoA antibody (Santa Cruz Biotechnology, Santa Cruz, Calif). The region containing RhoA was visualized using an ECL Western blotting luminal reagent (Santa Cruz Biotechnology).

Animal Preparation

Yorkshire male pigs (obtained from Kyudo, Tosu, Japan; aged 2 to 3 months and weighing 25 to 30 kg) were used. They were sedated with ketamine hydrochloride (12.5 mg/kg IM), euthanized with a lethal dose of sodium pentobarbital, and exsanguinated. The heart was excised. The coronary arteries at the IL-1β-treated and control sites were carefully dissected and cleaned of any perivascular tissue. For all experimental protocols, the endothelium was denuded by gently rubbing the luminal surface with a cotton swab and cut into rings approximately 4 mm in length. The rings were mounted vertically between hooks in a 20 mL capacity organ bath that was filled with physiological salt solution at 37°C. Isometric tension was measured by a force transducer. Each preparation was stretched in a stepwise manner to an optimal length at which the force induced by 62 mmol/L KCl became maximal and constant. The contractile responses to increasing concentrations of PDBu were examined in the presence and absence of hydroxyfasudil (3 μmol/L). The contractile responses were normalized and expressed in percent of the maximal contractions induced by 62 mmol/L KCl.

Measurement of GTPγS-Induced Ca²⁺ Sensitization in Permeabilized Coronary Smooth Muscle

A small strip of coronary smooth muscle was obtained from each arterial segment, mounted between a hook and a force transducer, and permeabilized by saponin. Then, the contractile responses to increasing concentrations of Ca²⁺ were examined in the presence and absence of GTPγS (10 μmol/L) in specimens from the spastic and control segments. The contracting solution contained various concentrations of CaCl₂ in addition to the relaxing solution. The contractions were expressed as percentage of the maximal response to 10 μmol/L Ca²⁺.

Western Blot Analysis for Membrane Translocation of PKC Isoforms

PKC has several isoenzymes. We reported that α-, β₁-, δ, and ζ-isozymes are expressed in the porcine coronary artery. Coronary segments from the spastic and control sites were dissected and cut into rings approximately 4 mm in length. Western blotting for PKC isoforms was performed. As a control, one ring was frozen after incubation in PSS, and as an activated preparation, another ring stimulated with serotonin (1 μmol/L) was frozen 5 minutes after exposure to the monoamine. These segments were minced with scissors into small pieces in ice-cold buffer, homogenized with a Polytron on ice, and then centrifuged at 100 000g for 30 minutes at 4°C. The cytosolic and membrane fractions were derived from the supernatant and pellet, respectively. All samples were boiled for 5 minutes and either used immediately or stored frozen at -20°C. Tissue extracts were subjected to SDS-PAGE as previously described. The antibodies used included rabbit PKC isoform-specific primary antibodies (Sigma, St. Louis, Mo, and Research & Diagnostic Antibodies, Berkley, Calif) and mouse PKC isoform-specific primary antibodies (Transduction Laboratory, Lexington, Ky). The extent of translocation of each PKC isoform from the cytosol to membrane fraction was used as a marker of the enzyme activity.

In Vivo Experiment

Two weeks after the operation, the animals were again anesthetized and ventilated, and selective coronary arteriography was performed by carotid approach. During the experiments, heparin (3 000 U IV) was administered every 60 minutes, and ECGs (leads I, II, III, V₁, and V₆), along with mean arterial pressure and heart rate, were continuously monitored and recorded on a pen recorder. The cineangiograms were projected on a screen using a cineprojector and an end-diastolic frame was selected and printed. The coronary luminal diameters were measured with a caliper. The accuracy of our measurements were previously confirmed. The degree of vasoconstricting response of the coronary artery was expressed as the percent decrease in the luminal diameter from the control level. Coronary arteriography was performed under control conditions, 2 minutes after intracoronary serotonin (10 μg/kg IC), and 20 minutes after intracoronary PDBu (5 μg/kg IC) in the presence and absence of hydroxyfasudil (10 μg/kg IC), a specific inhibitor of Rho-kinase. Coronary diameter was measured at the segment treated with IL-1β as well as at the control segment of a comparable diameter.
Statistical Analysis

Results are expressed as mean ± SEM. Throughout the text, n represents the number of animals tested. One-way or repeated two-way ANOVA was performed to evaluate the global statistical significance where appropriate, and if a significant F value was found, Scheffé post-hoc test was performed to identify the difference among the groups. A P < 0.05 was considered to be statistically significant.

Results

Effect of Hydroxyfasudil and Involvement of PKC in Small G Protein-Mediated Contractions of Permeabilized Porcine Coronary Arteries

GTPγS (10 μmol/L)-induced contractions of permeabilized normal coronary arterial strips were abolished by hydroxyfasudil (3 μmol/L), but were not significantly inhibited by calphostin C (1 μmol/L) (Figure 1, and Figure I, available online at http://atvb.ahajournals.org).

Augmented PDBu-Induced Membrane Translocation of RhoA in Porcine Coronary Smooth Muscle Cells

The ratio of membrane-bound RhoA was significantly increased by the treatment with PDBu (0.01 μmol/L) (Figure 2).

Inhibitory Effect of Hydroxyfasudil on PDBu-Induced Hyperconstrictions in Vivo and on Enhanced Contractile Responses to PDBu of Spastic Coronary Arteries in Vitro

Two weeks after the operation, serotonin (10 μg/kg IC) repeatedly caused hyperconstriction at the IL-1β-treated site, as we previously reported (data not shown).5-11 PDBu (5 μg/kg IC) also caused hyperconstriction at the IL-1β-treated site, a consistent finding with our past report (Figure 3). The pretreatment with hydroxyfasudil (10 μg/kg IC) did not significantly change the baseline heart rate or blood pressure (data not shown). Hydroxyfasudil significantly inhibited the PDBu-induced coronary hyperconstriction at the IL-1β-treated site in vivo, whereas its inhibitory effect was not evident at the control site (Figure 2). Contractions of spastic coronary segments to PDBu were significantly enhanced as compared with those of control segments (online Figure II, available online at http://atvb.ahajournals.org). The inhibitory effect of hydroxyfasudil (3 μmol/L) was greater in the spastic segments than in the control segments (Figure II).

Augmentation of GTPγS-Induced Ca2+-Sensitization in the Spastic Coronary Segments

The contractile responses to increasing concentrations of Ca2+ were examined in the presence and absence of GTPγS (10 μmol/L). GTPγS enhanced Ca2+-induced contractions in both the control and the spastic site, whereas the extent of the enhancement was greater in the spastic site than in the control site (Figure 4). In this series of experiments, the Ca2+-sensitizing effect of GTPγS was noted only at 0.3 μmol/L of Ca2+ in the control site (Figure 4).

Western Blot Analysis for PKC Translocation

Western blot analysis showed that among the 4 PKC isoforms examined, only PKCd in the spastic site was translocated from the cytosol to the membrane fraction on stimulation by serotonin (Figure 5).

Discussion

The major findings of this study in the spastic coronary segment of our porcine model were: (1) PDBu-induced contraction was significantly inhibited by hydroxyfasudil, a
specific Rho-kinase inhibitor, both in vivo and in vitro, whereas GTPγS-induced contraction was not inhibited by calphostin C, a specific PKC inhibitor, in vitro; (2) Rho-mediated Ca\(^{2+}\) sensitization of coronary smooth muscle was significantly enhanced; and (3) among the PKC isoforms, only PKC\(\gamma\) was involved in the coronary hypercontractions.

These results demonstrate that PKC and Rho-kinase coexist on the same intracellular signaling pathway with PKC located upstream on Rho-kinase, and that Rho/Rho-kinase–mediated pathway is essential for spasm occurrence and may be regulated by PKC\(\gamma\). Figure 6 shows our hypothesis for the intracellular mechanisms of coronary artery spasm.

### Interaction between PKC and Rho-Kinase in the Intracellular Signaling Pathway for Coronary Smooth Muscle Hypercontraction

We previously demonstrated that both PKC and Rho-kinase are substantially involved in agonists-induced coronary spasm in our porcine model.\(^{5,7-9,11-15}\) However, it remains to be elucidated whether there is an interaction between PKC and Rho-kinase in the intracellular signaling pathway for coronary smooth muscle hypercontraction. In the present study, GTPγS-induced contractions were abolished by hydroxyfasudil but were not inhibited by calphostin C in intact porcine coronary strips (Figure 1), suggesting that Rho/Rho-kinase–mediated pathway does not regulate PKC activities. In addition, the membrane translocation of RhoA was significantly increased by PDBu (Figure 2), suggesting that Rho/Rho-kinase–mediated pathway is regulated by PKC activities. We thus consider that PKC is one of the upstream regulators of Rho/Rho-kinase activation. Recently, several studies have suggested the interaction between PKC and Rho-kinase.\(^{23-28}\) Fu et al\(^{24}\) reported that PDBu-induced contraction of permeabilized rabbit pulmonary artery was not inhibited by Y-27632, another Rho-kinase inhibitor. Their observation seems different from our present findings. We consider that the concentration of PDBu they used (20 \(\mu\)mol/L) was too...
PKCδ was translocated from the cytosol to membrane fractions on stimulation by serotonin (Figure 5). Khalil et al.\textsuperscript{13} reported that phenylephrine caused Ca\textsuperscript{2+}-independent translocation of PKCe to the membrane and PKCδ to the cytosol, suggesting that Ca\textsuperscript{2+}-independent vascular smooth muscle contraction is associated with plasmalemmal translocation of PKC. Horowitz et al.\textsuperscript{14} also reported that PKCe produced contraction of a single, permeabilized ferret aorta smooth muscle cell. Therefore, Ca\textsuperscript{2+}-independent PKC isoforms, such as PKCe, might be involved in the regulation of smooth muscle contraction. PKC isoforms involved in the contractile response may be varied depending on the agonists, tissues, and animal species used. Our present results suggest that the serotonin-induced hypercontractions of porcine coronary arteries involve Ca\textsuperscript{2+}-independent PKC activation, especially that of PKCδ. Thus, PKCδ may be the key PKC isoform for coronary spasm, at least in our porcine model.

### Upregulation of Rho/Rho-Kinase–Mediated Pathway in Spastic Porcine Coronary Artery

Previously, we demonstrated that the enhanced and sustained MLC monophosphorylation and the appearance of MLC diphosphorylation are distinctive phenomena of coronary artery spasm\textsuperscript{6,9} and those enhanced MLC phosphorylations are caused by the inhibition of MLCPh by upregulated Rho-kinase.\textsuperscript{11} Indeed, the upregulation of Rho-kinase plays a key role in the molecular mechanisms of coronary artery spasm in our porcine model.\textsuperscript{11–15} On the other hand, PKC-mediated pathway is also an important mechanism of the spasm.\textsuperscript{5,7} However, there is little evidence of which is more essential for agonist-induced coronary spasm. In the present study, GTPγS-induced Ca\textsuperscript{2+} sensitization was upregulated in the spastic segment (Figure 4), whereas under control conditions, the proportion of PKCδ in the membrane and cytosol fraction was not different between the control and spastic site (Figure 5). These results suggest that Rho-kinase is more essential than PKC in agonist-induced coronary spasm in our porcine model. In a clinical setting, it is difficult to inhibit PKCδ alone because there is no specific PKCδ inhibitor presently available, and if nonspecific PKC inhibitor is used, it could cause various adverse effects because PKCs are involved in a variety of physiological cellular functions. Therefore, the present results suggest that Rho-kinase inhibitors may be more useful than PKC inhibitors for the treatment of coronary artery spasm.

### Limitations of the Present Study

Several limitations should be mentioned for the present study. First, the present study was performed in a porcine model but not in humans. Thus, our observations need to be confirmed in humans in a future study. Second, although hydroxyfasudil significantly inhibited PDBu-induced coronary spasm in vivo, it cannot be ruled out that other Rho-kinase effectors might also be involved in the inhibitory effect of hydroxyfasudil.

In conclusion, the present study demonstrates that PKC and Rho-kinase coexist on the same intracellular signaling pathway, with the former located upstream on the latter. PKCδ and Rho-kinase appear to be the key molecules for coronary spasm in our porcine model. The strategy to inhibit Rho-
kinase rather than PKC may be a more specific and useful treatment for the treatment of coronary artery spasm.

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Figure I:
A representative tracing of the developed force of a permeabilized coronary artery strip. GTPγS caused contractions at the fixed submaximal Ca\textsuperscript{2+} concentration (pCa = 7.0), and addition of hydroxyfasudil completely abolished the contractile response.
Figure II:
Inhibitory effect of hydroxyfasudil on contractile responses to increasing concentrations of PDBu in the control and spastic porcine coronary segments. Results are expressed as the mean ± SEM.