Involvement of Ca\textsuperscript{2+} Sensitization in Ropivacaine-induced Contraction of Rat Aortic Smooth Muscle

Jingui Yu, M.D.,* Yasuyuki Tokinaga, M.D.,† Toshiyuki Kuriyama, M.D.,‡ Nobuhiko Uematsu, M.D.,§ Kazuhiro Mizumoto, M.D.,|| Yoshiro Hatano, M.D.#

**Background:** The mechanisms of amino-amide local anesthetic agent-induced vasoconstriction remain unclear. The current study was designed to examine the roles of the protein kinase C (PKC), Rho kinase, and p44/42 mitogen-activated protein kinase (p44/42 MAPK) signaling pathways in calcium (Ca\textsuperscript{2+})-sensitization mechanisms in ropivacaine-induced vascular contraction.

**Methods:** Endothelium-denuded rat aortic rings, segments, and strips were prepared. The cumulative dose–response relations of contraction and intracellular Ca\textsuperscript{2+} concentration to ropivacaine were tested, using isometric force transducers and a fluorometer, respectively. The dose-dependent ropivacaine-induced phosphorylation of PKC and p44/42 MAPK and the membrane translocation of Rho kinase were also detected using Western blotting.

**Results:** Ropivacaine induced a dose-dependent biphasic contractile response and an increase in intracellular Ca\textsuperscript{2+} concentration of rat aortic rings, increasing at concentrations of 3 × 10\textsuperscript{-5} M to 3 × 10\textsuperscript{-4} M and decreasing from 10\textsuperscript{-3} M to 3 × 10\textsuperscript{-5} M, with a greater tension/intracellular Ca\textsuperscript{2+} concentration ratio than that induced with potassium chloride. The contraction was attenuated in a dose-dependent manner, by the PKC inhibitors bisindolylmaleimide I and calphostin C, the Rho kinase inhibitor Y 27632, and the p44/42 MAPK inhibitor PD 098059. Ropivacaine also induced an increase in phosphorylation of PKC and p44/42 MAPK, and membrane translocation of Rho kinase in accordance with the contractile responses, which were also significantly inhibited by bisindolylmaleimide I and calphostin C, Y 27632, and PD 098059, correspondingly.

**Conclusion:** These findings demonstrated that PKC-, Rho kinase-, and p44/42 MAPK–mediated Ca\textsuperscript{2+}-sensitization mechanisms are involved in the ropivacaine-induced biphasic contraction of rat aortic smooth muscle.

Many in vitro and in vivo studies have elucidated that amino-amide local anesthetic agents such as lidocaine, bupivacaine, mepivacaine and ropivacaine exhibit biphasic vascular effects, to varying extents: vasoconstriction at low concentrations and decrease of contraction or even vasodilation at high concentrations. The primary results in our current in vitro study demonstrated that these local anesthetics also exhibit biphasic vascular effects in rat aortic smooth muscle, with a strongest contractile response to ropivacaine (fig. 1). However, the molecular mechanism of the amino-amide local anesthetic agent–induced vasoconstriction remains unclear. Because ropivacaine is a new, long-acting, amino-amide local anesthetic and evokes potent vasoconstriction, regardless of the route of administration, we chose ropivacaine as representative of amino-amide local anesthetics to study their mechanism of vasoconstriction.

Smooth muscle contraction involves complex mechanisms, mainly calcium (Ca\textsuperscript{2+})–dependent and Ca\textsuperscript{2+} independent (or Ca\textsuperscript{2+}-sensitization) mechanisms. It is well known that potassium chloride (KCl)–induced smooth muscle contraction is mediated solely by Ca\textsuperscript{2+}-dependent mechanisms, i.e., via extracellular Ca\textsuperscript{2+} influx through L-type Ca\textsuperscript{2+} channels, without involvement of Ca\textsuperscript{2+}-sensitization mechanisms. The primary results of our study demonstrated that the ropivacaine-induced force/intracellular Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]\textsubscript{i}) ratio in rat aortic smooth muscle is higher than that in response to KCl (figs. 1B and 2A), suggesting that, in addition to the Ca\textsuperscript{2+}-dependent mechanism, Ca\textsuperscript{2+} sensitization is likely also involved in ropivacaine-induced vascular contraction.

Ca\textsuperscript{2+}-sensitization mechanisms refer primarily to protein kinase C (PKC)-, Rho kinase-, and p44/42 mitogen-activated protein kinase (p44/42 MAPK)–mediated signaling pathways, which play an important role in maintaining and regulating vascular tension. The current study is designed to investigate the role of Ca\textsuperscript{2+}-sensitization mechanisms, including PKC, Rho kinase, and p44/42 MAPK, in mediating ropivacaine-induced vasoconstriction by measurement of ropivacaine-induced contraction and the activation of PKC, Rho kinase, and p44/42 MAPK in rat aortic smooth muscle.

**Materials and Methods**

**Measurement of Isometric Tension**

The protocol was approved by the Animal Care and Use Committee of Wakayama Medical University (Wakayama City, Japan). Tissue preparation and tension measurement were performed as described previously. In brief, male Wistar rats (300–400 g) were anesthetized with halothane and were exsanguinated by bleeding from the common carotid artery. The descending thoracic aorta was harvested, and eight endothelium-denuded rings (3–4 mm in length) were prepared from each rat. The aortic rings were mounted to isometric force transducers (Nihondenki-sanei Co., Tokyo, Japan) and incubated in Krebs bicarbonate solution (consisting

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*Research Fellow, †Graduate Student, ‡Staff Anesthesiologist, †‡Assistant Professor, †§Professor and Chairman, Department of Anesthesiology, ‡§Staff Anesthesiologist, Department of Surgical Operating Center, Wakayama Medical University.

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Address reprint requests to Dr. Hatano, Department of Anesthesiology, Wakayama Medical University, 811-1 Kimidura, Wakayama City, 641-0012, Japan. Address electronic mail to: yhatano@wakayama-med.ac.jp. Individual article reprints may be purchased through the Journal Web site, www.anesthesiology.org.
of 118.2 mM sodium chloride, 4.6 mM KCl, 2.5 mM calcium chloride, 1.2 mM monobasic potassium phosphate, 1.2 mM magnesium sulfate heptahydrate, 24.8 mM sodium hydrogen carbonate, and 10.0 mM dextrose) maintained at 37°C and gassed continuously with a mixture of 95% oxygen–5% carbon dioxide (pH 7.4). The solution contained the cyclooxygenase inhibitor indomethacin (10⁻⁶ M) and the nitric oxide synthase inhibitor L-nitroarginine methyl ester (10⁻⁴ M) to prevent the release of endogenous prostaglandin I₂ and nitric oxide, respectively, from any residual endothelium. The rings were equilibrated for 1 h at a resting tension of 3 g, overall contractile responsiveness was assessed with KCl (3×10⁻² M), and complete removal of the endothelium was confirmed with acetylcholine (10⁻⁵ M). The tension experiment was performed with several subtype protocols. The local anesthetic agent–induced contractile responses were expressed as a percentage of the KCl (3×10⁻² M)–induced contraction.

The cumulative dose–response relations of the rings to ropivacaine, bupivacaine, mepivacaine, and lidocaine (3×10⁻⁵ M to 10⁻² M) were examined to compare their vascular effects. Subsequent doses of agents were administered after the previous dose elicited a sustained and stable contraction for 3–5 min. One ring from each animal randomly underwent the cumulative dose administration of one local anesthetic agent once.

The cumulative dose (3×10⁻⁵ M to 10⁻² M)–contractile response to ropivacaine was tested in the presence of the PKC inhibitors bisindolylmaleimide I (competing with adenosine triphosphate for binding to the catalytic domain, 10⁻⁶ M, 5×10⁻⁶ M, and 10⁻⁵ M) and calphostin C (competing with phorbol 12-myristate 13-acetate or...
diglyceride for binding to the regulatory domain, \(10^{-7} \text{M}, 5 \times 10^{-7} \text{M}, \) and \(10^{-6} \text{M}\), the Rho-kinase inhibitor Y 27632 \((5 \times 10^{-7} \text{M}, 10^{-6} \text{M}, \) and \(5 \times 10^{-6} \text{M}\), or the p44/42 MAPK inhibitor PD 098059 \((10^{-5} \text{M}, 5 \times 10^{-5} \text{M}, \) and \(10^{-4} \text{M}\). The effects of the combination of these inhibitors \((\text{bisindolylmaleimide I } [10^{-6} \text{M}] + \text{PD 098059} [5 \times 10^{-7} \text{M}] + \text{Y 27632} [10^{-5} \text{M}])\). The intimal surface of the muscle strip was illuminated with a 10-μM solution at 50 Hz at alternating excitation wavelengths of 340 and 380 nm, and the amount of fluorescence at 510 nm induced by 340 nm excitation \((F_{340})\) and that in-duced at 500 nm were measured. All of these inhibitors were delivered to the rings 15 min before the application of ropivacaine and maintained until the end of the measurement (preadministration). One ring from each animal was randomly challenged by only one concentration of an inhibitor or one combination of inhibitors, followed by the cumulative dose–administration of ropivacaine.

In another group of rings, \((\text{bisindolylmaleimide I } [5 \times 10^{-6} \text{M}], \text{calpastatin C} [5 \times 10^{-7} \text{M}], \) Y 27632 \([10^{-5} \text{M}]), \) and \(\text{PD 098059} [5 \times 10^{-5} \text{M}]\) were administered after a sustained ropivacaine \((3 \times 10^{-4} \text{M})\)-induced contraction had been achieved to observe their inhibitory effects on ropivacaine-elicited contraction (postadministration). One ring from each animal was randomly treated with only one inhibitor after receiving a single dose of ropivacaine.

Measurement of Intracellular \(Ca^{2+}\) Concentration

Endothelium-denuded aortic segments were treated with a \(10^{-5} \text{M} \) acetoxyethyl ester of fura-2 solution for 6–9 h at room temperature and were then fixed to a fluorometer \((\text{CAF}-110, \text{Japan Spectroscopic, Tokyo, Japan})\). The intimal surface of the muscle strip was illuminated at 50 Hz at alternating excitation wavelengths of 340 and 380 nm, and the amount of fluorescence at 510 nm induced by 340 nm excitation \((F_{340})\) and that induced by 380 nm excitation \((F_{380})\) were measured. The ratio \(F_{340}/F_{380}\) was used to indicate the [\(Ca^{2+}\)]\(_{i}\). The ratio \(F_{340}/F_{380}\) induced by \(3 \times 10^{-2} \text{M} \) KCl was measured first, and the values were considered as the reference \((100\%)\). Thereafter, the cumulative dose \((3 \times 10^{-5} \text{M} \text{to} \times 10^{-3} \text{M})\)–response of the ratio \(F_{340}/F_{380}\) to ropivacaine was tested and was expressed as a percentage of the reference values.

Detection of Protein Kinase Activation

The phosphorylation of PKC and p44/42 MAPK, and membrane translocation of Rho kinase (Rock-2) were detected using Western blotting with specific antibodies, as described previously. \(^{13–15}\) Briefly, the endothelium-denuded rat aortic strips (approximately 3.5 cm in length) were treated randomly with ropivacaine at concentrations of \(3 \times 10^{-5} \text{M}, 10^{-4} \text{M}, 3 \times 10^{-4} \text{M}, 10^{-3} \text{M}, \) or \(3 \times 10^{-3} \text{M}\) for 20 min, respectively, to measure the dose–response relation of ropivacaine-induced phosphorylation of PKC and p44/42 MAPK and Rho-kinase membrane translocation. Some strips were pretreated with bisindolylmaleimide I \((10^{-5} \text{M})\), calpastatin C \((10^{-6} \text{M})\), Y 27632 \((5 \times 10^{-6} \text{M}), \) or PD 098059 \((10^{-4} \text{M})\) for 15 min before challenge by ropivacaine \((3 \times 10^{-4} \text{M})\). Each animal provided only one aortic strip, and each strip was treated randomly with only one concentration of ropivacaine in the presence or absence of each inhibitor.

The agent-treated aortic strips were quickly frozen with dry ice and homogenized in lysis buffer \((50 \text{mM HEPES, pH } 7.5, 1 \text{mM Triton X-100, } 50 \text{mM sodium chloride, } 50 \text{mM sodium fluoride, } 5 \text{mM EDTA, } 5 \text{mM sodium pyrophosphate, } 1 \text{mM phenylmethylsulfonyl fluoride, } 1 \text{mM sodium orthovanadate, } 10 \mu\text{g/ml leupeptin, and } 20 \mu\text{g/ml aprotinin})\). Homogenates were centrifuged at 15,000 g for 15 min at 4°C. The supernatant was collected for the detection of PKC and p44/42MAPK phosphorylation. For the measurement of Rho-kinase membrane translocation, the homogenates were centrifuged at 13,000 g for 3 min at 4°C, and the supernatant was collected and then centrifuged at 100,000 g for 30 min at 4°C. The supernatant (cytosolic fraction) was removed, and the pellet (membrane fraction) was resuspended using the same buffer. The protein concentration of each sample was determined using the bicinchoninic acid method.

Equal amounts of total protein \((20–30 \mu\text{g})\) were used for every sample in each experiment. Proteins were separated by sodium-dodecyl-sulfate polyacrylamide gel electrophoresis and were transferred to nitrocellulose membrane. The membrane was treated with anti-PKC (1:1,000), anti-phospho-PKC (pan, βIIISer660, 1:1,000), anti-p44/42 MAPK (1:2,000), anti-phospho-p44/42 MAPK (Thr/Tyr204, 1:2,000), or anti–Rock-2 (1:1,000) antibodies, as appropriate, for 2 h, followed by incubation with horseradish peroxidase–conjugated antibody \((1:2,000)\) for 1 h at room temperature. Immunoreactive bands were detected using chemiluminescence \((\text{Amer}-\text{sham Pharmacia Biotech, Piscataway, NJ})\) and were assessed with ATTO Lane Analyzer 10H1.02 \((\text{ATTO Densi}-\text{tograph Software Library, Tokyo, Japan})\). The values for phosphorylation of PKC and p44/42 MAPK were expressed as a percentage of the density of total PKC and total p44/42 MAPK bands, respectively. The amount of Rock-2 in the membrane fraction was expressed as a percent of total Rock-2 value \((i.e., \text{membrane fraction plus cytosolic fraction})\). The sample size \((n)\) value represents the number of aortic strips from the same number of rats for each concentration of ropivacaine.

Materials

Ropivacaine was a kind gift from AstraZeneca (Osaka, Japan). Lidocaine, bupivacaine, mepivacaine, bisindolylmaleimide I, calpastatin C, and PD 098059 were purchased from Sigma-Aldrich Fine Chemicals (St. Louis, MO). Y 27632 was provided by Calbiochem-Novabio-chem Corporation (San Diego, CA). Polyclonal antibod-
Fig. 3. Effects of the protein kinase inhibitors on the resting tension (A and B) and ropivacaine (Rop)-induced sustained contraction (C and D) of rat aortic smooth muscle. The tension was measured using isometric force transducers. Endothelium-denuded rings were equilibrated for 1 h at a resting tension of 3 g and then exposed to different concentrations of bisindolylmaleimide I (Bis I) for 15 min (A). The statistical data for the effects of all of the inhibitors used on resting tension are presented in B. The resting tension (3 g) was considered as 100% (n = 6). A bolus of ropivacaine (3 × 10^{-4} M) was applied to the rings to induce a sustained contraction, and Bis I was then delivered (C). The statistical data for the effects of all of the inhibitors used on sustained ropivacaine-induced contraction are presented in D. Ropivacaine-induced contraction was expressed as a percent of the 30 mM potassium chloride-induced contraction. ** P < 0.01 versus control (n = 6). Calp C = calphostin C; PD = PD 098059; Y = Y 27632.

**A**Fig. 3. Effects of the protein kinase inhibitors on the resting tension (A and B) and ropivacaine (Rop)-induced sustained contraction (C and D) of rat aortic smooth muscle. The tension was measured using isometric force transducers. Endothelium-denuded rings were equilibrated for 1 h at a resting tension of 3 g and then exposed to different concentrations of bisindolylmaleimide I (Bis I) for 15 min (A). The statistical data for the effects of all of the inhibitors used on resting tension are presented in B. The resting tension (3 g) was considered as 100% (n = 6). A bolus of ropivacaine (3 × 10^{-4} M) was applied to the rings to induce a sustained contraction, and Bis I was then delivered (C). The statistical data for the effects of all of the inhibitors used on sustained ropivacaine-induced contraction are presented in D. Ropivacaine-induced contraction was expressed as a percent of the 30 mM potassium chloride-induced contraction. ** P < 0.01 versus control (n = 6). Calp C = calphostin C; PD = PD 098059; Y = Y 27632.

**B**Fig. 3. Effects of the protein kinase inhibitors on the resting tension (A and B) and ropivacaine (Rop)-induced sustained contraction (C and D) of rat aortic smooth muscle. The tension was measured using isometric force transducers. Endothelium-denuded rings were equilibrated for 1 h at a resting tension of 3 g and then exposed to different concentrations of bisindolylmaleimide I (Bis I) for 15 min (A). The statistical data for the effects of all of the inhibitors used on resting tension are presented in B. The resting tension (3 g) was considered as 100% (n = 6). A bolus of ropivacaine (3 × 10^{-4} M) was applied to the rings to induce a sustained contraction, and Bis I was then delivered (C). The statistical data for the effects of all of the inhibitors used on sustained ropivacaine-induced contraction are presented in D. Ropivacaine-induced contraction was expressed as a percent of the 30 mM potassium chloride-induced contraction. ** P < 0.01 versus control (n = 6). Calp C = calphostin C; PD = PD 098059; Y = Y 27632.

**C**Fig. 3. Effects of the protein kinase inhibitors on the resting tension (A and B) and ropivacaine (Rop)-induced sustained contraction (C and D) of rat aortic smooth muscle. The tension was measured using isometric force transducers. Endothelium-denuded rings were equilibrated for 1 h at a resting tension of 3 g and then exposed to different concentrations of bisindolylmaleimide I (Bis I) for 15 min (A). The statistical data for the effects of all of the inhibitors used on resting tension are presented in B. The resting tension (3 g) was considered as 100% (n = 6). A bolus of ropivacaine (3 × 10^{-4} M) was applied to the rings to induce a sustained contraction, and Bis I was then delivered (C). The statistical data for the effects of all of the inhibitors used on sustained ropivacaine-induced contraction are presented in D. Ropivacaine-induced contraction was expressed as a percent of the 30 mM potassium chloride-induced contraction. ** P < 0.01 versus control (n = 6). Calp C = calphostin C; PD = PD 098059; Y = Y 27632.

**D**Fig. 3. Effects of the protein kinase inhibitors on the resting tension (A and B) and ropivacaine (Rop)-induced sustained contraction (C and D) of rat aortic smooth muscle. The tension was measured using isometric force transducers. Endothelium-denuded rings were equilibrated for 1 h at a resting tension of 3 g and then exposed to different concentrations of bisindolylmaleimide I (Bis I) for 15 min (A). The statistical data for the effects of all of the inhibitors used on resting tension are presented in B. The resting tension (3 g) was considered as 100% (n = 6). A bolus of ropivacaine (3 × 10^{-4} M) was applied to the rings to induce a sustained contraction, and Bis I was then delivered (C). The statistical data for the effects of all of the inhibitors used on sustained ropivacaine-induced contraction are presented in D. Ropivacaine-induced contraction was expressed as a percent of the 30 mM potassium chloride-induced contraction. ** P < 0.01 versus control (n = 6). Calp C = calphostin C; PD = PD 098059; Y = Y 27632.

Amino-amide Local Anesthetic–induced Contractile Responses of Rat Aortic Smooth Muscle

All of the amino-amide local anesthetics tested induced dose-dependent, biphasic contraction of rat aortic endothelium-denuded rings. The rank order for degree of vasoconstriction was ropivacaine > bupivacaine > mepivacaine > lidocaine. The concentrations required to achieve the maximal contraction are 10^{-4} M, 3 × 10^{-4} M, 3 × 10^{-3} M, and 3 × 10^{-3} M for ropivacaine, bupivacaine, mepivacaine, and lidocaine, respectively (fig. 1). Because ropivacaine has strongest contractile properties against phospho-PKC (pan, βIIIser660), p44/42 MAPK, and phospho-p44/42MAPK (Thr/Tyr204) were supplied by Cell Signaling Technology Inc. (Beverly, MA). Polyclonal antibodies against PKC (H-300) and Rock-2 and the secondary antibody labeled with horse-radish peroxidase were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). All other reagents for the experiments were of analytical grade.

**Data Analysis**

All data are presented as mean ± SD. The sample size (n value) represents the number of rats from which aortic rings were taken for each protocol. Differences among anesthetic agents at the same concentration and the effects of different concentrations of inhibitors on ropivacaine at same concentration were tested by one-way analysis of variance followed by an unpaired Student t test with a Bonferroni correction. The ropivacaine-induced dose-dependent changes in [Ca^{2+}]_i and protein kinase activation were analyzed by two-way analysis of variance for repeated measures and a paired Student t test with a Bonferroni correction for post hoc comparisons. P values less than 0.05 were considered statistically significant.

**Results**

Amino-amide Local Anesthetic–induced Contractile Responses of Rat Aortic Smooth Muscle

All of the amino-amide local anesthetics tested induced dose-dependent, biphasic contraction of rat aortic endothelium-denuded rings. The rank order for degree of vasoconstriction was ropivacaine > bupivacaine > mepivacaine > lidocaine. The concentrations required to achieve the maximal contraction are 10^{-4} M, 3 × 10^{-4} M, 3 × 10^{-3} M, and 3 × 10^{-3} M for ropivacaine, bupivacaine, mepivacaine, and lidocaine, respectively (fig. 1). Because ropivacaine has strongest contractile properties...
among the amino-amide local anesthetics, we focused on the elucidation of its contractile mechanism in the following experiments.

**Ropivacaine-elicited Increase in Intracellular Ca\(^{2+}\) Concentration**

Ropivacaine elicited a dose-dependent biphasic change in \([\text{Ca}^{2+}]_i\) (F340/F380), which was consistent with the tension pattern: increase from concentration of \(3 \times 10^{-5} \text{ M}\) to \(3 \times 10^{-4} \text{ M}\), decrease from concentration of \(10^{-3} \text{ M}\) to \(3 \times 10^{-5} \text{ M}\) (figs. 2A and B). The ratio of ropivacaine-induced force/\([\text{Ca}^{2+}]_i\) was higher than that induced by KCl.

**Effects of PKC, Rho-Kinase, and p44/42 MAPK Inhibitors on Ropivacaine-Induced Contraction of Rat Aortic Smooth Muscle**

The PKC inhibitors bisindolylmaleimide I and calphostin C, the Rho-kinase inhibitor Y 27632 (Y; C), and the p44/42 MAPK inhibitor PD 098059 (PD; D) on the ropivacaine (Rop)-induced, dose-dependent, cumulative contraction of rat aortic smooth muscle. Endothelium-denuded rings were randomly pretreated with the above-mentioned inhibitors at different concentrations for 15 min and were then challenged by ropivacaine in a cumulative dose manner. The tension was measured using isometric force transducers and was expressed as a percent of the 30 mm potassium chloride–induced contraction. *\(P < 0.05\), **\(P < 0.01\) versus control (\(n = 6\)).

**Ropivacaine-induced Phosphorylation of PKC and p44/42 MAPK and Membrane Translocation of Rho Kinase**

Anti-PKC and anti–phospho-PKC antibodies can recognize several PKC subtypes, including PKC-\(\alpha\), -\(\beta1\), -\(\beta2\), -\(\varepsilon\), -\(\eta\), and -\(\delta\), with molecular weights between 78 and 85 kd, which appeared as double bands in the current study (figs. 6A and B). Bands with molecular weights of 150, 44, and 42 kd were confirmed as Rock-2 (fig. 6C), p44, and p42 MAPK (fig. 6D), respectively. No differences were detected in the density of the PKC or p44/42 MAPK or the total density of Rock-2 (i.e., membrane

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**Fig. 4.** Effects of the protein kinase C inhibitor bisindolylmaleimide I (Bis I; A), calphostin C (Calp C; B), the Rho-kinase inhibitor Y 27632 (Y; C), and the p44/42 MAPK inhibitor PD 098059 (PD; D) on the ropivacaine (Rop)-induced, dose-dependent, cumulative contraction of rat aortic smooth muscle. Endothelium-denuded rings were randomly pretreated with the above-mentioned inhibitors at different concentrations for 15 min and were then challenged by ropivacaine in a cumulative dose manner. The tension was measured using isometric force transducers and was expressed as a percent of the 30 mm potassium chloride–induced contraction. *\(P < 0.05\), **\(P < 0.01\) versus control (\(n = 6\)).
Fig. 5. Effects of combining bisindolylmaleimide I (Bis I; 10^{-6} \text{ M}), Y 27632 (Y; 5 \times 10^{-7} \text{ M}), and PD 098059 (PD; 10^{-5} \text{ M}) on the ropivacaine (Rop)-induced, dose-dependent, cumulative contraction of rat aortic smooth muscle. Endothelium-denuded rings were randomly pretreated with one of the four combinations for 15 min and then challenged by ropivacaine in a cumulative dose manner. The tension was measured using isometric force transducers and was expressed as a percent of the 30 mM potassium chloride–induced contraction. *P < 0.05, **P < 0.01 versus control (n = 6).

fraction plus cytosolic fraction) bands at each dose of ropivacaine. However, the densities of the phosphorylated PKC and phosphorylated p44/42 MAPK bands and the density of the Rock-2 band in the membrane fraction changed in accord with the ropivacaine treatment: increasing at 3 \times 10^{-5} \text{ M} and 10^{-4} \text{ M}, reaching the maximum level at 3 \times 10^{-4} \text{ M}, and decreasing from 10^{-3} \text{ M} to 3 \times 10^{-3} \text{ M} (figs. 6A-D), which was consistent with the contractile response. The presence of bisindolylmaleimide I (10^{-5} \text{ M}), calphostin C (10^{-6} \text{ M}), Y 27632 (5 \times 10^{-6} \text{ M}), or PD 098059 (10^{-4} \text{ M}) almost completely abrogated the ropivacaine (3 \times 10^{-4} \text{ M})-induced increase in the densities of the phosphorylated PKC and p44/42 MAPK bands and the density of the Rock-2 band in the membrane fraction, respectively (figs. 6A-D).

Discussion

The main findings of the current study are as follows: (1) Amino-amide local anesthetics induced a dose-dependent, biphasic response of rat aortic endothelium-denuded rings. Ropivacaine induced the strongest contraction among them, which was attenuated in a dose-dependent manner by the PKC inhibitors bisindolylmaleimide I and calphostin C, the Rho-kinase inhibitor Y 27632, and the p44/42 MAPK inhibitor PD 098059, respectively. (2) Ropivacaine also elicited biphasic changes in [Ca^{2+}]_i consistent with the tension pattern, with a higher force/[Ca^{2+}]_i than that induced by KCl. (3) Ropivacaine induced an enhancement in the phosphorylation of PKC and p44/42 MAPK and membrane translocation of Rho kinase in accord with the contractile response that was each significantly attenuated by bisindolylmaleimide I, calphostin C, Y 27632, and PD 098059.

The biphasic vascular effect is a common characteristic of amino-amide local anesthetic agents that has been well examined in vivo and in vitro. Meanwhile, the intensity of vasoconstriction and the concentration required to reach the peak level of contraction are different among this class of drugs.1–8 However, these studies have primarily focused on the contractile phenomenon and their clinical effects. Little is known about their contraction mechanism. Based on the results of the current and other studies, ropivacaine possesses potent and typical biphasic vascular effects and is therefore the object of the current investigation of the molecular mechanisms of amino-amide local anesthetic–induced vasoconstriction.

An increasing body of evidence has elucidated the role of Ca^{2+}-dependent and Ca^{2+}-sensitization mechanisms in mediating smooth muscle contraction, and the involvement of the PKC, Rho-kinase, and p44/42 MAPK signaling pathways in Ca^{2+} sensitization.12–15 During the process of activation, PKC and p44/42 MAPK become phosphorylated,21,22,15 and Rho kinase translocates to the cell membrane.14 Consequently, activation of PKC, p44/42 MAPK, and Rho kinase can be assessed by the detection of PKC and p44/42 MAPK phosphorylation23–25 and Rho-kinase membrane translocation,26,27 respectively. The activated PKC and Rho kinase then primarily phosphorylate the myosin light chain phosphatase to attenuate its activity, thus attenuating the dephosphorylation of phosphorylated myosin light chain 20, and potentiating the activation of actomyosin adenosine triphosphatase and eliciting contraction without affecting [Ca^{2+}]_i.13,14 The activated p44/42 MAPK (stimulated mainly by tyrosine kinase, but also partly by PKC) phosphorylates caldesmon and attenuates the inhibition of actomyosin adenosine triphosphatase activity, consequently enhancing smooth muscle contraction, without changing [Ca^{2+}]_i.15 The higher force/[Ca^{2+}]_i ratio in the ropivacaine-induced vascular contraction compared with that in response to KCl in the current study supports the role of mediation of Ca^{2+} sensitivity in the ropivacaine-induced vascular contraction. The roles of PKC, Rho kinase, and p44/42 MAPK in the mediation of the ropivacaine-induced vasoconstriction were demonstrated by the findings of the current study that ropivacaine induced both vascular contraction and phosphorylation of PKC and p44/42 MAPK and membrane translocation of Rho kinase that were inhibited by the respective PKC, Rho-kinase, and p44/42 MAPK inhibitors. The greater extent of the inhibition of the com-
bined inhibitors on the contraction compared with the single inhibitors also suggests that all three of these protein kinase–mediated pathways are involved in the ropivacaine-induced contraction. The efficacy of the inhibitors on the depression of the activities of ropivacaine was confirmed by the observation of their negligible inhibitory effects on resting tension, but their significant inhibition of the sustained ropivacaine-induced contraction and the ropivacaine-elicited activation of protein kinases.

In the process of regulating smooth muscle contraction, all signaling pathways are not absolutely independent of each other. Some cross-talk or common downstream effectors may exist among these pathways. For example, myosin light chain phosphatase is phosphorylated by both PKC and Rho kinase, and p44/42 MAPK can be phosphorylated and activated by both PKC and tyrosine kinase. Therefore, the cross-talk between PKC and p44/42 MAPK may be helpful to explain that the inhibition of contraction by the combination of bisindolylmaleimide I and PD 098059 was less than that of bisindolylmaleimide I and Y 27632 or Y 27632 and PD 098059 in the current study.

The greater extent of the inhibition of the ropivacaine-induced contraction by each of the inhibitors at the highest concentrations used in the current study does not mean that each of the kinase-mediated signaling pathways is capable of dominating the contraction. One of the possible reasons could be the specificity of inhibitors. None of these inhibitors or antagonists can be considered absolutely specific, i.e., the higher the concentration is, the relatively lower the specificity is. Therefore, it is possible that the inhibitors also interrupt other signaling cascades at high concentration in addition to their inhibitory effects on their specific targets.

An interesting finding of the current study is that increasing the concentration to higher than 10^−3 m decreased both the ropivacaine-induced contraction and activation of PKC, Rho kinase, and p44/42 MAPK, indicating that the inhibition of PKC, Rho-kinase, and p44/42 MAPK activation by high concentrations of ropivacaine could at least be one of the causes of the decrease in contraction. However, the mechanism by which the activation of these kinases is limited by high concentrations of ropivacaine could not be explained by the current study. We postulate that ropivacaine at high concentrations could trigger relaxation mechanisms and/or inhibit contraction mechanisms. From another perspective, the current study demonstrated that PKC, Rho kinase, and p44/42 MAPK each contribute as medi-
tors of the ropivacaine-induced contraction. However, whether ropivacaine activates these kinases directly or by activating their upstream effectors, such as tyrosine kinase, or any combination thereof, will require further investigations.

The clinically relevant concentration of ropivacaine is approximately 0.25–0.75% (approximately equivalent to 0.8–2.4 × 10⁻² M). At this concentration, ropivacaine elicits local blanching by intradermal injection, decreases epidermal blood flow by epidermal injection, and reduces cutaneous capillary blood flow by subcutaneous infiltration, supporting the impact of ropivacaine on vasoconstriction. However, such concentrations of ropivacaine would not elicit any vascular effect in the current study. We suggest that this discrepancy between our study and others could be caused by several aspects. First, injected ropivacaine may be diluted by interstitial fluid to concentrations that exert vasoconstriction, although the exact concentration of locally injected ropivacaine has not been measured. Second, the rat aorta used in the current study is a conductance vessel, which primarily functions for conducting blood. Therefore, the aortic contractile response to ropivacaine would likely be different from that in resistant blood vessels and capillaries. The extent of the vasoconstriction produced in the aorta does not always represent the situation of the whole organism in vivo. Third, the vascular sensitivity to ropivacaine may not be same in different species. Another important cause may be the great differences in experimental conditions between in vivo and in vitro studies.

In summary, the findings of the current study demonstrated that ropivacaine induced a dose-dependent biphasic contraction, phosphorylation of PKC and p44/42 MAPK, and membrane translocation of Rho kinase in rat aortic smooth muscle and suggests that PKC, p44/42 MAPK, and Rho kinase are all involved in the ropivacaine-induced vascular contraction.

References

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