Rho-Rho kinase pathway is involved in the regulation of myogenic tone and pump activity in isolated lymph vessels

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Hosaka, Kayoko, Risuke Mizuno, and Toshio Ohhashi. Rho-Rho kinase pathway is involved in the regulation of myogenic tone and pump activity in isolated lymph vessels. Am J Physiol Heart Circ Physiol 284: H2015–H2025, 2003; 10.1152/ajpheart.00763.2002.—To evaluate whether or not Rho-Rho kinase pathway is involved in the regulation of mechanical activity of lymph vessels, effects of Y-27632 and okadaic acid on lymph pump activity and myogenic, pressure- and agonist-induced tone were examined in isolated rat lymph vessels. Y-27632 caused a significant dilation with a cessation of the lymph pump activity. Y-27632 also produced a dose-related dilation of the lymph vessels precontracted by norepinephrine (NE), U-46619, or 80 mM KCl. Okadaic acid significantly constricted the lymph vessels and reduced the frequency of the lymph pump activity. Okadaic acid also produced a dose-related constriction of the lymph vessels precontracted by NE or U-46619. The Y-27632-induced decrease of the frequency of lymph pump activity was significantly reversed by the pretreatment with okadaic acid. In the presence of Y-27632, the pressure-mediated tone of the lymph vessel was significantly decreased. On the other hand, okadaic acid significantly increased the pressure-mediated tone. These findings suggest that Rho kinase and myosin phosphatase activity in lymphatic smooth muscles may contribute to the regulation of lymph pump activity and may be also involved in the control of myogenic pressure- and agonist-induced tone.

myogenic tone of lymphatic smooth muscles regulates elastic behavior of the wall and then results in control of the active pump activity. In addition, an increased Ca2+ influx through the membrane and release of membrane-bound and intracellularly stored Ca2+ are also known to contribute to the agonist-induced contractions of lymphatic smooth muscles (2, 24). Recent studies have revealed important roles for the small GTPase Rho and its effector Rho-associated kinase (Rho kinase) in Ca2+-independent regulation of smooth muscle contractions. The Rho-Rho kinase pathway modulates the level of phosphorylation of the myosin light chain of myosin II, mainly through the inhibition of myosin phosphatase, and contributes to agonist-induced Ca2+ sensitization in smooth muscle contractions (8, 29). In addition, in the agonist-induced contractions of vascular smooth muscles, the Rho-Rho kinase pathway is also directly involved in phosphorylating myosin light chain, resulting in augmentation of the contractions (16).

Little information exists, however, regarding the crucial roles of the Rho-Rho kinase pathway in the regulation of agonist- or pressure-mediated tone and active pump activity of lymph vessels. Thus to clarify the crucial roles of the Rho-Rho kinase pathway in the mechanical activity of lymphatic smooth muscles, we examined the effects of the selective Rho kinase inhibitor Y-27632 (8, 37) and the selective myosin phosphatase inhibitor okadaic acid (10, 32) on the myogenic tone and active pump activity of isolated iliac rat lymph microvessels.

MATERIALS AND METHODS

Seven-week-old male Wistar rats (~200 g body wt, n = 84; SLC) were used for the studies. The rats were housed in an environmentally controlled vivarium and fed a standard pelleted diet and water ad libitum. All experimental protocols were approved by the Animal Ethics Committee of Shinshu University School of Medicine in accordance with the principles and guidelines of the Physiological Society of Japan on Animal Care.

Lymphatic preparation. Rats were anesthetized with pentobarbital sodium (50 mg/kg ip). After an abdominal incision...
was performed, the iliac afferent lymph vessels with their lymph nodes were excised and placed in a petri dish containing cold (4°C) Krebs bicarbonate solution. The Krebs solution contained (in mM) 120.0 NaCl, 5.9 KCl, 2.5 CaCl₂, 1.2 MgSO₄, 1.2 NaH₂PO₄, 5.5 glucose, and 25.0 NaHCO₃. With the use of microsurgical instruments and an operating microscope, the lymph vessels (n = 84, 250 μm maximum diameter and 3 mm long) were isolated and then transferred to a 10-ml organ chamber, with two glass micropipettes containing the Krebs bicarbonate solution.

After each lymph vessel was mounted on a pipette (proximal) and secured with sutures, the perfusion pressure was raised to 4 cmH₂O to flush out and clear the vessel. The distal end of the lymph vessel was then mounted to the outflow micropipette (distal). The proximal and distal micropipettes were connected through Tygon tubing with a 50-ml syringe and with a stopcock, respectively. The Krebs bicarbonate solution, while being maintained at a PO₂ level of ~50 mmHg (pH 7.4 ± 0.01) and bubbled with 5% CO₂-95% N₂, was perfused extraluminally over the lymph vessels within the organ bath. The flow rate of the perfused solution was kept at 10 ml/min. In the experiments described in the present study, by using the organ chamber (10 ml) and extraluminal perfusion system (flow rate at 12 ml/min), we needed at least 1 min to obtain maximum concentration of the drugs in the organ chamber and to wash out the drugs from the chamber. Thus the periods of delay in the lymphatic responses to a single dose of the drugs are always observed at the starting or ending point of the administration of drugs (see Figs. 1 and 6).

After the lymph vessel was cannulated, the chamber was transferred to the stage of an intravital microscope (model BH-2, Olympus). The lymph vessels were then warmed slowly to 37°C and allowed to equilibrate for 60 min.

Measurement of diameter of lymph vessels. The images of the lymph microvessels were obtained through the use of an objective lens (×4), a photo eyepiece lens (×3.3), and a monochrome charge-coupled device camera (model KCB-270A, KOKOM). Changes in the diameter of the lymph microvessels were manually and automatically measured with a custom-designed diameter-detection device with the use of an edge-detection method (27). The changes were recorded on both a videocassette recorder (Toshiba) and a direct-writing oscillograph (Recti 8K, Sanei-Sokki). The intraluminal pressure in the lymph vessels was kept at 5 cmH₂O by elevating a 50-ml syringe connected to the inflow tubing, whereas the outflow tubing was closed with a stopcock throughout the experiments. The pressure was optimal to produce active pump activity of the isolated rat lymph vessel (19).

Experimental protocol. To evaluate functional viability of the lymphatic endothelial cells, acetylcholine (ACh, 10 μM) was first perfused extraluminally over all of the lymph vessels before starting the experiments. In the first experimental protocol, the dose-dependent effects of Y-27632 (1, 3, and 6 μM), a selective Rho kinase inhibitor (8, 37), on lymph pump activity of the lymph vessels with or without intact endothelium were investigated. To remove the lymphatic endothelial cells, 200 μl of air were gently perfused into the lumen of the lymph vessels for 3 min and then the lumen was flushed with the Krebs solution for 1 min. The lymph vessels without intact endothelium also exhibited lymph pump activity, whereas the vessels did not show ACh-induced negative chronotropic effect on the lymph pump activity.

In the second protocol, we investigated the effects of Y-27632 (1, 3, and 6 μM) on the myogenic high-KCl Krebs solution-, norepinephrine (NE)-, or U-46619-induced tone (80 mM, 10 μM, and 0.1 mM, respectively) in some of the lymph vessels. The high-KCl solution was replaced by replacement of NaCl in the normal Krebs-bicarbonate solution with equimolar amounts of KCl.

In the third protocol, the effects of Y-27632 (1, 3, and 6 μM) on lymph pump activity of the lymph vessels were investigated in the absence or presence of 30 μm nitric oxide (NO) synthase inhibitor, N⁵-endo nitr-o-l-arginine methyl ester (l-NAME) (22), a 10 μM cyclooxygenase inhibitor indomethacin (20), or 1 μM glibenclamide, a selective ATP-sensitive K⁺ channel blocker (21). Because the lymph pump activity was strongly regulated by endogenous NO (9, 22, 23, 26, 39, 41), prostaglandins (PGs) (14, 21), and ATP-sensitive K⁺ channels (21, 39), we examined whether or not endogenous NO, PGs, and ATP-sensitive K⁺ channels are involved in the Y-27632-induced responses of lymph pump activity. The lymph vessels were pretreated with the various blockers for 30 min before the extraluminal perfusion of Y-27632 through the organ chamber.

In the fourth protocol, effects of okadaic acid (0.1 and 1 μM), a selective myosin phosphatase inhibitor (10, 32), on the lymph pump activity were investigated in the pressurized perfusion of drugs with or without intact endothelium. Also, the effects of okadaic acid on myogenic-, 10 μM NE-, or 0.1 μM U-46619-induced tone of the lymph vessels were investigated in some lymph vessels. To determine the interaction between the Rho kinase and phosphatase in the lymphatic smooth muscles, the Y-27632-induced responses of lymph pump activity in the pressurized lymph vessels were also examined in the absence or presence of 0.1 μM okadaic acid.

In the fifth protocol, effects of 3 μM Y-27632 or 0.1 μM okadaic acid on the increasing of the intraluminal pressure-mediated tone were investigated. Changes in the maximum and minimum diameters (Dₘₐₓ and Dₘᵢₙ, respectively) of the lymph vessels and the frequency of lymph pump activity were measured in the absence and presence of 3 μM Y-27632 or 0.1 μM okadaic acid when the intraluminal pressure increased stepwise, ranging from 3 to 9 cmH₂O by a step pressure of 2 cmH₂O. At the end of each experiment, Ca²⁺-free Krebs solutions containing 1 mM EGTA-mediated maximal dilation were obtained at the corresponding intraluminal pressures to normalize the extent of changes in Dₘₐₓ (19).

Drugs. All salts (Wako), Y-27632 (Mitsubishi Pharma), ACh chloride (Daichi-seiyaku; Tokyo, Japan), glibenclamide (RBI; Natick, MA), okadaic acid (Kamiya Biomedical; Seattle, WA), EGTA (Daijindo), U-46619 (Cayman; Ann Arbor, MI), as well as l-NAME, indomethacin, and NE (Sigma; St. Louis, MO) were used in the present study. The glibenclamide and okadaic acid were diluted with DMSO, and the concentrations of DMSO used did not affect the myogenic tone and active pump activity of the isolated lymph vessels. The drug concentrations were expressed as the final concentration in the organ chamber. All salts and drugs were prepared on the day of the experiment.

Statistical analyses. Dₘₐₓ, Dₘᵢₙ, amplitude (Dₘₐₓ – Dₘᵢ₉), and frequency (times/min) of lymph pump activity in the lymph vessels were measured. The drug-induced responses of lymph pump activity were expressed as a percentage of the extent of inhibition of the pump activity before and after the administration of drugs. The averaged frequency (times/min) of lymph pump activity during the drug-induced response was normalized by that obtained before the application of drugs. Dₘₐₓ, Dₘᵢₙ, and percent frequency were normalized by Dₘₐₓ, Dₘᵢₙ, and frequency, respectively, before the drugs were applied. In the cases to evaluate the effects of Y-27632 or okadaic acid on the NE-, U-46619- or high-potassium solution-induced constriction in lymph vessels, percent changes in the diameters were obtained by a
percentage of each agent-induced precontraction level. The data are presented as means ± SE, and n indicates the number of vessels. Significant differences (P < 0.05) were determined with the use of one-way ANOVA, followed by Duncan's post hoc test, and unpaired and paired Student's t-test, as appropriate.

RESULTS

Effects of Y-27632 on lymph pump activity in lymph vessels with or without endothelium. Figure 1A shows representative recordings of Y-27632 (1, 3, and 6 μM)-induced responses of lymph pump activity in the lymph vessels with intact endothelium. Y-27632 induced a dose-related dilation of the lymph vessels with a cessation of lymph pump activity. The Y-27632-induced period of cessation increased dose dependently. Figure 1, B–D, summarizes the effects of Y-27632 on %Dmax, %Dmin, and percent frequency, respectively, of lymph pump activity in the lymph vessels with intact endothelium (n = 8). Y-27632 significantly increased the %Dmax. The Y-27632-mediated maximum dilations were independent of the concentration of agonist used. On the other hand, the %Dmin of the lymph vessels increased dose dependently. Thus the %Dmax and %Dmin values of the lymph pump activity at 6 μM Y-27632 were 116.0 ± 3.4% (not significant vs. from 1


**Effects of Y-27632 on NE-, U-46619- or high-potassium solution-induced tone of lymph vessels.** Figure 2, A and B, shows representative tracings of dose-related responses of Y-27632 (1, 3, and 6 μM) in the lymph vessels precontracted by NE (10 μM) and U-46619 (0.1 μM), respectively. Y-27632 caused a dose-related dilation on the myogenic NE- and U-46619-induced tone of the lymph vessels. The open and solid bars in Fig. 2C summarize the Y-27632-induced dilation in the presence of 10 μM NE (n = 5) and 0.1 μM U-46619 (n = 5), respectively.

Figure 3A shows a representative tracing of dose-related responses of Y-27632 (1, 3, and 6 μM) on high-

![Diagram](image-url)
potassium solution-induced tone of the lymph vessel. Y-27632 also caused a dose-related dilation on the high-potassium-induced precontraction. Figure 3B shows the summarized data of effects of Y-27632 on the high-potassium-induced tone ($n = 5$).

Effects of L-NAME, indomethacin, or glibenclamide on Y-27632-induced responses of lymph pump activity in lymph vessels with intact endothelium. To determine the involvement of endogenous NO, vasodilator prostanoids, or activation of ATP-sensitive K$^+$ channels in the Y-27632-induced responses, the Y-27632-induced dilation and reduction of the frequency of the lymph pump activity were investigated before or after the treatment with 30 $\mu$M L-NAME, 10 $\mu$M indomethacin, or 1 $\mu$M glibenclamide. Table 1 shows the summarized data for the 6 $\mu$M Y-27632-induced changes in the %D$_{max}$ and percent frequency of the lymph pump activity before or after the treatment with the inhibitors. Treatment with L-NAME, indomethacin, or glibenclamide did not significantly affect the 6 $\mu$M Y-27632-induced dilation and reduction of the frequency of the lymph pump activity.

Effects of okadaic acid on lymph pump activity in lymph vessels with or without intact endothelium. Figure 4A shows a representative recording of okadaic acid (1 $\mu$M)-mediated effects on lymph pump activity in Fig. 4. A: representative trace of the effect of okadaic acid (1 $\mu$M) on the lymph pump activity of the lymph vessel and the arrow indicates the starting point for the superfusion of okadaic acid. Effects of okadaic acid (0.1 and 1 $\mu$M) on $D_{max}$ (B), $D_{min}$ (C), frequency (D), and amplitude ($D_{max} - D_{min}$) (E) of lymph pump activity of the lymph vessels ($n = 4$). Open and solid bars show the values before and after the application of the concentration of okadaic acid, respectively. *$P < 0.05$, significant difference from before the application of okadaic acid.

<table>
<thead>
<tr>
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<th>%D$_{max}$</th>
<th>%Frequency</th>
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<tr>
<td>Control</td>
<td>119.2 ± 3.0</td>
<td>78.4 ± 3.8</td>
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<tr>
<td>L-NAME (30 $\mu$M)</td>
<td>119.0 ± 4.2*</td>
<td>82.5 ± 4.7*</td>
</tr>
<tr>
<td>Indomethacin (10 $\mu$M)</td>
<td>122.8 ± 5.0</td>
<td>73.9 ± 6.9</td>
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<tr>
<td>Glibenclamide (1 $\mu$M)</td>
<td>120.8 ± 3.8</td>
<td>72.1 ± 7.1</td>
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Values are means ± SE; $n$, no. of vessels. L-NAME, N$^\omega$-nitro-L-arginine methyl ester; %D$_{max}$, percentage of maximum diameter. *Insignificant difference from control.

Table 1. Effects of L-NAME, indomethacin, or glibenclamide on 6 $\mu$M Y-27632-induced changes in %D$_{max}$ of lymph vessels and %frequency of lymph pump activity

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the pressurized lymph vessel. The concentration of okadaic acid caused a significant constriction and reduction of the frequency of the lymph pump activity in the lymph vessel at ~10 min after starting extraluminal superfusion of the drug. Figure 4, B–E, shows the effects of 0.1 and 1 μM okadaic acid on the \( D_{\text{max}} \), \( D_{\text{min}} \), frequency, and \( D_{\text{max}} - D_{\text{min}} \), respectively, of lymph pump activity in the lymph vessels (\( n = 4 \)). Okadaic acid significantly decreased the \( D_{\text{min}} \) (Fig. 4C) and frequency (Fig. 4D) of active pump activity in the lymph vessels. In contrast, the concentration of okadaic acid caused no significant effect on the \( D_{\text{max}} \). The 1 μM okadaic acid-induced reduction of the \( D_{\text{min}} \) and frequency were 80.7 ± 7.2 μm (\( n = 4 \), \( P < 0.05 \) vs. before the application; 96.0 ± 7.1 μm) and 17.8 ± 1.0 min\(^{-1} \) (\( n = 4 \), \( P < 0.05 \) vs. before the application; 19.3 ± 0.6 min\(^{-1} \)), respectively.

The lymph vessels without intact endothelium also demonstrated the okadaic acid-induced responses in similar to those obtained with the lymph vessels with intact endothelium. Thus the \%\( D_{\text{max}} \), \%\( D_{\text{min}} \), and percent frequency of lymph pump activity in the lymph vessels without endothelium in the presence of 1 μM okadaic acid were 83.1 ± 4.8% (\( n = 4 \), not significant from the intact endothelium; 91.6 ± 5.7, \( n = 4 \)), 87.1 ± 4.9% (\( n = 4 \), not significant from the intact endothelium; 83.8 ± 2.2%, \( n = 4 \)), and 92.3 ± 7.9% (\( n = 4 \), not significant from the intact endothelium; 92.0 ± 2.9%, \( n = 4 \)), respectively.

**Effects of okadaic acid on NE- and U-46619-induced tone of lymph vessels.** Figure 5, A and B, shows representative tracings of dose-related responses of okadaic acid (0.1 and 1 μM) in the lymph vessels precontracted by NE (10 μM) and U-46619 (0.1 μM), respectively. Okadaic acid caused dose-related constriction on the agonist-mediated precontraction of the lymph vessels.

The bars in Fig. 5C show summarized the okadaic acid-induced constriction on NE and U-46619 precontraction of the lymph vessels (\( n = 4 \) for both groups).

**Effects of okadaic acid on 1 μM Y-27632-induced responses of lymph pump activity in lymph vessels with intact endothelium.** To evaluate the interaction between the Rho kinase and phosphatase in the lymphatic smooth muscles, the effects of Y-27632 on lymph pump activity in the lymph vessels were investigated in the absence or presence of 0.1 μM okadaic acid. Figure 6A shows representative tracings of the effects of okadaic acid (0.1 μM) on the 1 μM Y-27632-induced responses of the lymph vessels. The Y-27632-induced cessation of the lymph pump activity was significantly reduced by the treatment with okadaic acid (0.1 M).

Figure 6, B–D, summarizes the effects of okadaic acid (0.1 μM) on the Y-27632-induced dilation (\( B \), \%\( D_{\text{max}} \) and \( C \), \%\( D_{\text{min}} \)) and reduction of the frequency (\( D \); percent frequency) of lymph pump activity in the lymph vessels with intact endothelium (\( n = 4 \)). The Y-27632-mediated reduction of the frequency was significantly suppressed by additional treatment with 0.1 μM okadaic acid (Fig. 6D).

**Effects of Y-27632 or okadaic acid on pressure-induced tone of lymph vessels.** Figure 7 shows representative tracings of the effects of Y-27632 (3 μM; A) or okadaic acid (0.1 μM; B) on the lymph pump activity and pressure-dependent diameters of the lymph vessels. In the normal Krebs solution containing no agonists, the stepwise increase of the intraluminal pressure ranging from 3 to 9 cmH\(_2\)O caused a significant increase of the \( D_{\text{min}} \) (from 115.8 ± 5.2 μm at 3 cmH\(_2\)O to 164.1 ± 5.1 μm at 9 cmH\(_2\)O, \( n = 12 \)) and the frequency (from 19.8 ± 1.0 min\(^{-1} \) at 3 cmH\(_2\)O to 29.3 ± 0.7 min\(^{-1} \) at 9 cmH\(_2\)O, \( n = 12 \)) of the lymph pump activity. On the other hand, the stepwise increase of

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**Fig. 5.** Representative tracings of effects of okadaic acid (0.1 and 1 μM) on the 10 μM NE (A) and 0.1 μM U-46619 (B) induced precontractions of the lymph vessels. C: open and solid bars show okadaic acid-induced %changes in the diameter in the presence of NE (\( n = 4 \)) and U-46619 (\( n = 4 \)), respectively. *P < 0.05, significant difference from the 0.1 μM okadaic acid-induced responses; †significant difference from the values shown in the open bars (NE-induced changes in diameter).
the intraluminal pressure from 5 to 9 cmH₂O caused no or few changes in the \(D_{\text{max}}\) (from 224.0 ± 8.1 μm at 5 cmH₂O to 217.4 ± 8.1 μm at 9 cmH₂O, \(n = 12\)).

In the presence of 3 μM Y-27632, the oscillatory lymph pump activity disappeared and the stepwise increasing of the intraluminal pressure (3–9 cmH₂O) caused a slight dilation of the diameter in the lymph vessel (Fig. 7A). Thus the maximal diameters obtained at the intraluminal pressures of 3–9 cmH₂O in the presence of Y-27632 were significantly larger than those obtained with the normal Krebs solution (control). In the presence of 0.1 μM okadaic acid, the oscillatory lymph pump activity also disappeared and the stepwise increasing of intraluminal pressure ranging from 3 to 9 cmH₂O caused more dilation of the maximal diameters obtained at the corresponding intraluminal pressures.

Figure 8, A and B, summarized the effects of 3 μM Y-27632 and 0.1 μM okadaic acid on relative changes in the maximal diameters obtained at the corresponding intraluminal pressures ranging from 3 to 9 cmH₂O. The relative changes in maximal diameter were measured by a percentage of each passive diameter produced by Ca²⁺-free Krebs solution, including 1 mM EGTA. The relative changes in the maximal diameters in the presence of okadaic acid were significantly less than those obtained in the absence of okadaic acid (control). In the presence of Ca²⁺-free Krebs solution containing 1 mM EGTA, the stepwise increasing of the intraluminal pressure caused significant increases of the maximal diameters obtained at the corresponding intraluminal pressures.

In contrast, in the case of 0.1 μM okadaic acid, the oscillatory lymph pump activity also disappeared and the stepwise increasing of intraluminal pressure ranging from 3 to 9 cmH₂O significantly increased the maximal diameters of the lymph vessels obtained at the corresponding pressures (Fig. 7B). The maximal diameters obtained at each intraluminal pressure in the presence of okadaic acid were significantly less than those produced in the normal Krebs solution (the control). In the presence of Ca²⁺-free Krebs solution containing 1 mM EGTA, the stepwise increasing of the intraluminal pressure caused significant increases of the maximal diameters obtained at the corresponding intraluminal pressures.
linear relationships between the intraluminal pressure and the relative changes in maximal diameter were negative (-0.91 ± 0.10 at a pressure from 5 to 9 cmH₂O) and positive (0.77 ± 0.24 at a pressure from 5 to 9 cmH₂O, P < 0.05 from the absence) in the absence and presence of okadaic acid, respectively.

**DISCUSSION**

The major findings of the present study are as follows. First, Y-27632, a selective Rho kinase inhibitor, elicits a significant dilation with a cessation of lymph pump activity in isolated pressurized lymph vessels with intact endothelium. Second, Y-27632 also causes a significant dilation in lymph vessels precontracted high-potassium solution, NE, or U-46619. Third, the removal of lymphatic endothelium or pretreatment with L-NAME, indomethacin, or glibenclamide produces no significant effect on the Y-27632-induced responses of lymph pump activity. Fourth, okadaic acid, a selective myosin phosphatase inhibitor, constricts the pressurized lymph vessels and reduces the frequency of the lymph pump activity. Fifth, okadaic acid also causes a significant constriction in lymph vessels precontracted by NE or U-46619. Sixth, pretreatment with 0.1 μM okadaic acid significantly inhibits the Y-27632 (1 μM)-mediated responses of lymph pump activity. Seventh, Y-27632 significantly decreases the pressure-induced passive diameter of the lymph vessels and then disappears the stretch-mediated contraction of lymphatic smooth muscles (Baylis effect). Finally, okadaic acid significantly increases the pressure-induced passive diameter of the lymph vessels and then disappears the stretch-mediated contraction of lymphatic smooth muscles. The present study is the first demonstration that the Rho-Rho kinase pathway.
is involved in the regulation of lymph pump activity of the lymph vessels and the myogenic-, pressure- or agonist-induced tone of the lymph vessels. The concentrations of Y-27632 and okadaic acid used in the present experiment are well known to inhibit selectively the Rho kinase and myosin phosphatase in vascular smooth muscles (6, 8, 10, 31, 32, 37). It is, however, noteworthy to take into account the nonspecific pharmacological effects of the concentrations of inhibitors used on lymphatic smooth muscles and endothelial cells. No one knows the nonspecific effects of the inhibitors in lymph vessels. In the future, further investigation will be needed to evaluate such nonspecific pharmacological effects of the inhibitors in lymph vessels.

**Effects of Y-27632 on lymph pump activity and agonist-induced tone of lymph vessels.** It is well known that spontaneous contractions of smooth muscles in the lymph vessels play an important role in active lymph transport mechanisms (1, 30). The rhythmic constriction and dilation of the lymph vessels were observed in many tissues and regulated by the myogenic activity of lymphatic smooth muscles (4, 9, 12, 14, 17, 19, 25, 38, 42). The spontaneous contractions of lymphatic smooth muscles are strongly regulated by Ca^{2+} spikes through an activation of slow inward current (3), resulting in an increase of Ca^{2+} influx through the plasma membrane. The increased intracellular Ca^{2+} may bind to calmodulin, and then the Ca^{2+}-calmodulin complex may activate myosin light chain kinase of lymphatic smooth muscles. The sophisticated mechanisms downstream of the increased intracellular Ca^{2+} in contraction of lymphatic smooth muscles remain to be fully understood. However, it is widely accepted that intracellular Ca^{2+} is a primary regulator of tonic and phasic contractions in the lymphatic smooth muscles (2). However, Ca^{2+}-independent contractions and Ca^{2+}-sensitization mechanisms of lymphatic smooth muscles remain unknown. In addition, differences in calmodulin and calmodulin-binding proteins and in Ca^{2+} sensitization in phasic and tonic contractions of lymphatic smooth muscles also remain to be clarified. In visceral smooth muscles (35), the phasic and tonic behavior of smooth muscles may be related to differences in content and isoform composition of contractile proteins and intracellular signaling pathways, including Ca^{2+}, calmodulin, and calmodulin-binding proteins that regulate the activities of contractile proteins (5, 7, 13, 28, 33, 36). The tonic smooth muscles also differ from phasic muscles in maintaining tonic contraction that is thought to be due to the "latch" cross bridges that produce economical force at a low actomyosin in ATPase activity (34, 40).

In the present study, Y-27632 caused a significant dilation of the lymph vessels and reduced the frequency of the lymph pump activity. The lymph vessels precontracted by NE, U-46619, or high-potassium solution (80 mM) also dilated dose dependently in response to Y-27632. The effective and selective concentrations of Y-27632 to inhibit Rho kinase and relaxations of vascular smooth muscles ranged from 1 to 10 \( \mu \text{M} \) (6, 18, 31, 37). The Y-27632 concentrations used (1–6 \( \mu \text{M} \)) in the present experiment may be reasonable to evaluate pivotal roles of Rho kinase in lymphatic smooth muscles and endothelial cells.

The suppressive action of Rho kinase-mediated inhibition myosin phosphatase produced by Y-27632 may contribute to the Y-27632-mediated dilation in the lymph vessels or the reduction of NE-, U-46619- or high-potassium-mediated precontraction of the lymph vessels in the present experiments. The findings suggest that the Rho-Rho kinase pathway may be involved in keeping the lymph pump activity and the myogenic- and agonist-mediated tone of lymphatic smooth muscles. The conclusion may be compatible with previous findings that Rho kinase activated by Rho inhibits myosin phosphatase activity in blood vessels, which enhances contractility of vascular smooth muscles (8, 15, 33) and that Y-27632 inhibits agonist-induced contraction of vascular smooth muscles (8). In addition, the pretreatment with Y-27632 caused a significant increase of the pressure-mediated maximal diameters in the lymph vessels. The findings suggest that the Rho-Rho kinase pathway may be also related to the regulation of pressure-mediated tone of lymphatic smooth muscles.

The Rho-Rho kinase pathway may be related, in part, to the maintenance of phasic contraction of lymphatic smooth muscles, because Y-27632 caused a cessation in the lymph pump activity. This conclusion also agrees with our previous studies (2, 26), in which Ca^{2+} spikes and the resultant increase of Ca^{2+} influx through the membrane elicit the phasic contractions of lymphatic smooth muscles. The conclusion either may be or strongly supported by a recent study suggested that the membrane depolarization-induced increase in intracellular Ca^{2+} concentration produces a contraction of vascular smooth muscles via Rho-associated kinase (18).

Recently, we have also reported that lymph pump activity of the lymph vessels was strongly regulated by endothelium-derived NO (19, 23, 24, 41), PGs (14, 21), and ATP-sensitive K^+ channels (19, 20, 23, 39). In the present study, removal of lymphatic endothelium caused no significant effect on the Y-27632-induced inhibitory responses of the lymph vessels. Pretreatment with an inhibitor of NO synthase, cyclooxygenase, or ATP-sensitive K^+ channels did not affect significantly the Y-27632-induced inhibitory responses of the lymph vessels. These findings suggest that Y-27632 has directly inhibited lymph pump activity in the lymph vessels with endothelium-independent mechanisms, and that NO, vasodilator PGs, and activation of ATP-sensitive K^+ channels did not contribute to the Y-27632-mediated inhibitory responses of the lymph vessels.

**Effects of okadaic acid on lymph pump activity and agonist-induced tone of lymph vessels.** Okadaic acid, a cytotoxic crystalline compound isolated from *Haliclona okadai*, is a selective inhibitor of myosin phosphatase in vascular smooth muscles (32). An inhibition of myosin phosphatase causes a reduction of dephosphor-
ylation of myosin light chain in the smooth muscles. Thus okadaic acid produces a potent contraction of vascular smooth muscles (10, 34). In the present study, okadaic acid significantly constricted and reduced the frequency of lymph pump activity in the pressurized lymph vessels. In addition, okadaic acid significantly constricted the lymph vessels precontracted by NE or U-46619. These findings also support our conclusion that myosin phosphatase activity in lymphatic smooth muscles may play an important role for the regulation of lymph pump activity and agonist-induced myogenic tone of the lymph vessels. However, the okadaic acid-mediated reduction of the frequency of lymph pump activity might not fit with the present findings that study the effects of Y-27632 in the frequency. Further investigations will be needed to evaluate the mode of interaction between Y-27632 and okadaic acid in the regulation of frequency of lymph pump activity.

By contrast, the 1 μM Y-27632-induced cessation of lymph pump activity in the lymph vessels was significantly reversed by the presence of 0.1 μM okadaic acid (Fig. 6). The finding strongly suggests that myosin phosphatase activity in the lymphatic smooth muscles may play an important role in keeping lymph pump activity in the lymph vessels. The reason why the treatment with 0.1 μM okadaic acid did not affect the Y-27632-mediated D_{min} of the lymph vessels remains unclear. Further investigation will be needed to evaluate in detail the mode of pharmacological and signal-transductional interaction between Y-27632 and okadaic acid in the lymphatic smooth muscles.

Effects of Y-27632 or okadaic acid on pressure-induced tone in lymph vessels. In the present study, the stepwise increasing of intraluminal pressure in the pressurized lymph vessels increased significantly the minimal diameters of the lymph vessels and augmented the frequency of lymph pump activity. On the other hand, D_{max} of the lymph vessels did not change significantly by the stepwise increase of the intraluminal pressure. The evidence may be related to the present finding that the maximum effect of Y-27632 is independent of the concentration used (Fig. 1). The %D_{max}, normalized by a percentage of maximal passive diameter in each lymph vessel that produced Ca^{2+}-free Krebs solution containing 1 mM EGTA, was ~80%. These findings were compatible to the conclusion of our previous study (22).

In the present experiments by using stimulation of the stepwise increase of intraluminal pressure, the D_{max} in the presence of Y-27632 were significantly larger than those obtained at each corresponding pressure in the normal Krebs solution (the control). In addition, the D_{max} in the presence of Y-27632 were significantly less than those obtained at each corresponding pressure in the of Ca^{2+}-free Krebs solution, including 1 mM EGTA. Thus the %D_{max} in the presence of Y-27632 were 95~98%. These results suggest that Y-27632 causes significantly an increase of the pressure-induced maximal diameters of the lymph vessels. Recently, it has been reported that Y-27632 plays a significant role for vascular myogenic tone in vivo and in vitro studies (6, 31). Thus Schubert et al. (31) reported that the intravascular pressure-induced tone of isolated rat tail small arteries was inactivated by 3 μM Y-27632. The effective concentrations of Y-27632 for the inactivation of the myogenic tone were ranging from 1 to 10 μM (6, 18, 31, 37). Thus the concentration of 3 μM Y-27632 used in the present study may be quite reliable. Therefore, the present findings suggest that Rho-kinase may be involved in the regulation of the pressure-induced tone in isolated lymph vessels. In addition, 3 μM Y-27632 also causes a significant reduction of the stretch-induced contractions of lymphatic smooth muscles.

In contrast, the pretreatment of 0.1 μM okadaic acid caused a significant decrease of the pressure-induced maximal diameters of the lymph vessels. The %D_{max} in the presence of okadaic acid was ~70%. In addition, 0.1 μM okadaic acid also produces a significant reduction of the stretch-induced contractions of lymphatic smooth muscles. The findings suggest that myosin phosphatase activity also plays an important role in the regulation of pressure-induced tone in isolated lymph vessels. The mode of action of okadaic acid in the reduction of the stretch-induced contractions remains unclear. Further investigation will be needed to evaluate the mode of action of okadaic acid in lymph vessels, including the nonspecific pharmacological action.

In conclusion, the Rho-Rho kinase pathway is involved in the regulation of active lymph pump activity of the rat iliac lymph microvessels and of the myogenic-, pressure- or agonist-induced tone of the lymph vessels.

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