ATP inhibits pump activity of lymph vessels via adenosine A₁ receptor-mediated involvement of NO- and ATP-sensitive K⁺ channels

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The transport of lymph depends on passive and active driving forces as well as the rate of lymph production in organs and tissues. The active driving forces, resulting from intrinsic pump activity, play a pivotal role in the centripetal propulsion of lymph (1, 31, 39). Neural (21), humoral (30), and mechanical factors (8, 22, 23) modify the rhythm and amplitude of lymphatic intrinsic pump activity. Recently, it has become clear that lymphatic endothelium-derived nitric oxide (NO) strongly contributes to regulation of lymphatic intrinsic pump activity. Thus NO causes relaxation of lymphatic smooth muscles and reduces lymphatic intrinsic pump activity (24, 26, 27, 35, 40, 45, 48).

ATP-sensitive K⁺ (KATP) channels have been found in the plasma membrane of cells, including vascular and nonvascular smooth muscles, and then became well known to play an important role in regulation of smooth muscle functions (37). KATP channels in the lymph vessels also play significant roles in control of intrinsic pump activity of the lymphatic smooth muscles (25–27, 44).

Lymphatic smooth muscles in some lymph vessels contain numerous mitochondria and glycogen granules, which may reflect high metabolic activity of lymphatic smooth muscles (32). The existence of blood capillaries within the walls of lymph vessels is related to the high metabolic activity (32). The presence of numerous mitochondria and glycogen granules leads to speculation that a large amount of ATP is produced in the smooth muscles of lymph vessels with intrinsic pump activity. ATP is also an important physiological substance, in that it regulates contraction and relaxation of smooth muscles in the vascular system (3, 38). ATP increased the frequency of spontaneous contractions of lymphatic smooth muscles in sheep (13) and guinea pigs (7, 50). In contrast, isolated and precontracted ring preparations of bovine (33), porcine (10), and canine (41) lymph vessels caused relaxation in response to ATP. Whether ATP can decrease intrinsic pump activity of lymph vessels has not been resolved.

Therefore, in the present study, we have attempted to reexamine the effects of ATP on intrinsic pump activity of isolated rat iliac lymph vessels with special reference to crucial roles of endogenous NO and KATP channels.

MATERIALS AND METHODS

Seven-week-old male Wistar rats (n = 112; Japan SLC) were housed in an environmentally controlled vivarium and fed a standard pellet 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 on animal care of the Physiological Society of Japan.

Lymphatic preparations. The rats were anesthetized with pentobarbital sodium (50 mg/kg ip) and exsanguinated. After an incision of the abdomen, the iliac afferent lymph vessels with their lymph nodes were excised and placed in a petri dish containing cold (4°C) Krebs-bicarbonate solution (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 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

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flush out and clear the vessel. Then, the distal end of the lymph vessel was mounted to the outflow micropipette (distal). The proximal and distal micropipettes were connected through Tygon tubing with a 50-ml syringe and a stopcock, respectively. The Krebs-bicarbonate solution, in which PO$_2$ was maintained at 56.7 ± 1.6 mmHg (n = 10) and pH at 7.4 ± 0.01 by bubbling with 5% CO$_2$-95% N$_2$ (15, 25–27), was perfused extraluminally over the lymph vessels within the organ chamber. Physiological salt solution with 5% CO$_2$-95% N$_2$ is also useful for studying lymphatic circulation (2, 36, 40, 49), because PO$_2$ in the lymphatic circulation is known to be ~25–40 mmHg, which is lower than that obtained with blood circulation in physiological conditions (9, 16).

The flow rate of the perfused solution was kept at 15 ml/min throughout the experiment. In the present experiments, using the chamber (10 ml) and extraluminal perfusion system (flow rate at 15 ml/min), we needed ≥1 min to obtain maximum concentrations of the drugs in the organ chamber and to wash out the drugs from the chamber (15, 25–27). Thus, to obtain dose-dependent responses of agonists, we circulated extraluminally the Krebs-bicarbonate solution containing each drug into the organ chamber for 3 min. After cannulation of the lymph vessel, the chamber was transferred to the stage of an intravital microscope (Nikon Microphoto). The lymph vessels were then warmed slowly to 37°C and allowed to equilibrate for 60 min.

**Measurement of lymph vessel diameter.** An objective lens (×4), a photo-eyepiece lens (×3.3), and a monochrome charge-coupled device camera (KOKOM KCB-270A) were used to obtain images of the lymph microvessels, which were displayed on a monochrome television monitor (Hamamatsu Photonics). Changes in the diameter of the lymph vessels were manually and automatically measured with a diameter-detection device with an edge-detection method (36). They were recorded on a videocassette recorder (Toshiba) and a direct-writing oscillograph (Sanei-Sokki, Recti 8K). The intraluminal pressure in the lymph vessels was kept at 5 cmH$_2$O by elevation of a 50-ml syringe connected to the inflow tubing; the outflow tubing was closed with a stopcock throughout the experiments. The pressure was optimal for production of intrinsic pump activity of the isolated rat lymph vessel (15, 23).

**Experimental protocols.** To evaluate functional viability of the lymphatic endothelial cells, 10$^{-5}$ M ACh was perfused extraluminally over all the lymph vessels before the experiments were started (15, 23). In the first experimental protocol, to evaluate ATP-induced desensitization of intrinsic pump activity of the lymph vessels, time- and dose-dependent effects of ATP (3 × 10$^{-8}$–10$^{-6}$ M, applied 3 times) on lymphatic pump activity were investigated for 30 min. Effects of αβ-methylene ATP (3 × 10$^{-8}$–10$^{-6}$ M) on lymphatic pump activity were also investigated. In the second experimental protocol, effects of ATP (3 × 10$^{-8}$ –10$^{-6}$ M) on intrinsic pump activity of the lymph vessels with or without intact endothelium were investigated. To remove the lymphatic endothelial cells, air (200 μl) was gently perfused into the lumen of the lymph vessels for 3 min, and then the lumen was flushed with the Krebs-bicarbonate solution for 1 min. The lymph vessels without intact endothelium also exhibited intrinsic pump activity, while the vessels did not show a ACh-induced negative chronotropic effect on lymphatic pump activity (15, 23). To determine histologically the removal of lymphatic endothelium by the air-perfusion method, the lymph vessels were fixed with 10% formalin solution, and the sections (4 μm) were stained with hematoxylin and eosin. In the third protocol, the effects of ATP (3 × 10$^{-8}$–10$^{-7}$ M) on lymphatic pump activity were investigated in the absence or presence of 3 × 10$^{-5}$ M Nω-nitro-l-arginine methyl ester (an inhibitor of NO synthase; L-NAME), 3 × 10$^{-5}$ M L-NAME + 10$^{-3}$ M l-arginine, 10$^{-5}$ M indomethacin (a cyclooxygenase inhibitor), or 10$^{-6}$ M glibenclamide (a selective K$_{ATP}$ channel blocker). These concentrations of the inhibitors have been known to produce selectively each specific pharmacological action in the isolated lymph vessels (24–27).

In the fourth protocol, the effects of ATP (3 × 10$^{-8}$–10$^{-6}$ M) on lymphatic pump activity were investigated in the absence or presence of 10$^{-4}$ M suramin (an antagonist of P2X and P2Y receptors) (3, 13, 50), 10$^{-7}$ and 3 × 10$^{-7}$ M 8-cyclopentyl-1,3-dipropylxanthine (DPCPX, a selective adenosine A$_2$ antagonist) (29, 38), or 3 × 10$^{-7}$ M 3,7-dimethyl-1-proparglyxanthine (DMPX, a selective adenosine A$_2$ antagonist) (29, 38). In the final protocol, the effects of 3 × 10$^{-8}$–10$^{-6}$ M adenosine on lymphatic pump activity were investigated in the absence or presence of 3 × 10$^{-8}$–10$^{-6}$ M L-NAME, 10$^{-6}$ M glibenclamide, or 3 × 10$^{-7}$ M DPCPX. The lymph vessels were pretreated with the various blockers and inhibitors for 30 min before extraluminal perfusion of ATP or adenosine through the organ chamber.

**Drugs.** All salts were obtained from Wako; ACh from Daiichiseiyaku (Tokyo, Japan); glibenclamide, DPCPX, and DMPX from Research Biologicals; and ATP, adenosine, αβ-methylene ATP, L-NAME, l-arginine, indomethacin, and suramin from Sigma. Glibenclamide was diluted with DMSO, and DPCPX, DMPX, and indomethacin were diluted with ethanol. The concentrations of DMSO and ethanol did not affect the myogenic tone and intrinsic pump activity of the isolated lymph vessels. Concentrations of drugs were expressed as the final concentration in the organ chamber. All salts and drugs were prepared on the day of the experiment. ATP, adenosine, and αβ-methylene ATP were kept on ice between applications, which seemed to inhibit degradation of the drugs (34).

**Statistical analyses.** The frequency (times/min) of intrinsic pump activity and minimum diameter (D$_{min}$, μm) and maximum diameter (D$_{max}$, μm) of the lymph vessels were measured. The drug-induced responses of intrinsic pump activity are expressed as relative changes in the frequency of pump activity and changes in D$_{min}$ and D$_{max}$ of the lymph vessels. Changes in the parameters during the drug-induced responses were normalized by each control value of the parameters obtained before application of the drugs (15, 27). The drug-induced cessation periods (s) of lymphatic pump activity express the maximum inhibitory time after perfusion of each drug (15). Values are means ± SE, and n indicates the number of vessels. Significant differences (P < 0.05) were determined by one-way ANOVA followed by Student-Newman-Keuls post hoc test and unpaired and paired Student’s t-test, as appropriate.

**RESULTS**

Effects of ATP and αβ-methylene ATP on intrinsic pump activity of lymph vessels. Figure 1A shows representative traces of the effects of 3 × 10$^{-8}$–10$^{-6}$ M ATP on intrinsic pump activity of the isolated lymph vessel. ATP caused dose-dependent dilation with a cessation of lymphatic pump activity (Fig. 1, B and C). The dose-dependent responses were obtained with three applications of ATP (3 × 10$^{-8}$–10$^{-6}$ M) at 30-min intervals. The percent frequencies of lymphatic pump activity with the first, second, and third applications of 10$^{-6}$ M ATP were 35.9 ± 6.0, 29.7 ± 5.1, and 23.4 ± 2.3%, respectively (n = 6), and the cessation periods of lymphatic pump activity with the first, second, and third applications of 10$^{-6}$ M ATP were 173.0 ± 30.8, 164.3 ± 22.6, and 183.8 ± 23.7 s, respectively (n = 6). There was no desensitization with repeated administration of ATP.

ATP significantly increased D$_{max}$ and D$_{min}$ in a dose-dependent manner, resulting in dilation of the isolated lymph vessels. Thus D$_{max}$ and D$_{min}$ induced by 10$^{-6}$ M ATP were 261.5 ± 17.1 μm [P < 0.05 vs. control (241.7 ± 18.3 μm), n = 6] and 261.5 ± 17.1 μm [P < 0.05 vs. control (119.2 ± 15.3 μm), n = 6], respectively (Fig. 1, D and E).

αβ-Methylene ATP (3 × 10$^{-8}$–10$^{-6}$ M) caused no significant effect on intrinsic pump activity of the isolated lymph vessels; thus the frequencies of pump activity before and after application of 10$^{-6}$ M αβ-methylene ATP were 22.7 ± 1.3
Effects of removal of the endothelium on ATP-induced responses.

Figure 2 shows cross-sectional photomicrographs of lymph vessels with and without intact endothelium. The layer of endothelium in the lymph vessel after perfusion with air was completely removed (Fig. 2B).

The lymph vessels without intact endothelium also exhibited intrinsic pump activity. Average frequencies of pump activity of lymph vessels were 20.0 ± 2.5 and 16.0 ± 1.0 min⁻¹ with and without endothelium, respectively (n = 7, P = NS).

Removal of the endothelium caused a complete reduction of ACh-induced negative ino- and chronotropic effects on lymphatic pump activity (data not shown). In contrast, removal of the endothelium produced a partial reduction of ATP-mediated dilation with a cessation of pump activity of the lymph vessels. Thus the inhibitory response mediated by 3 × 10⁻⁷–10⁻⁶ M ATP remains in the lymph vessels without endothelium (Fig. 2, C and D). Changes in percent frequency of lymphatic pump activity induced by 3 × 10⁻⁷ M ATP with and without endothelium were 43.6 ± 6.0% and 87.9 ± 4.2%, respectively (n = 7, P < 0.05). The
cessation periods of lymphatic pump activity induced by $3 \times 10^{-7}$ M ATP with and without endothelium were $137.9 \pm 22.7$ and $8.7 \pm 2.7$ s, respectively ($n = 7$, $P < 0.05$).

Changes in percent $D_{\text{max}}$ mediated by $3 \times 10^{-8}$–$10^{-7}$ M ATP were significantly reduced in lymph vessels without intact endothelium (Fig. 2E). Changes in percent $D_{\text{min}}$ mediated by $10^{-7}$–$10^{-6}$ M ATP were significantly reduced in lymph vessels without intact endothelium (Fig. 2F).

Effects of l-NAME, l-NAME + l-arginine, and indomethacin on ATP-induced responses. Figure 3A shows representative traces of $10^{-6}$ M ATP-induced inhibitory responses of lymphatic pump activity without l-NAME, with $3 \times 10^{-5}$ M l-NAME, or with $3 \times 10^{-5}$ M l-NAME + $10^{-3}$ M l-arginine. The ATP-induced dilation with cessation of lymphatic pump activity was significantly suppressed by pretreatment with $3 \times 10^{-5}$ M l-NAME. Treatment with l-NAME, however, did not reduce completely the $10^{-6}$ M ATP-mediated inhibitory responses. Additional treatment with $10^{-3}$ M l-arginine significantly restored the l-NAME-induced reduction of the ATP-mediated inhibitory response (Fig. 3, B and C). Changes in percent frequency of lymphatic pump activity induced by $10^{-6}$ M ATP without l-NAME, with l-NAME alone, and with l-NAME + l-arginine were $24.4 \pm 3.2\%$ ($n = 6$), $60.2 \pm 7.4\%$ ($n = 6$, $P < 0.05$ vs. without l-NAME), $P < 0.05$ vs. l-NAME + l-arginine), and $20.9 \pm 5.2\%$ ($n = 6$), respectively. The cessation periods of lymphatic pump activity induced by $10^{-6}$ M ATP without l-NAME, with l-NAME alone, and with l-NAME + l-arginine were $217.5 \pm 25.0$ s ($n = 6$), $45.7 \pm 24.4$ s ($n = 6$, $P < 0.05$ vs. without l-NAME), $P < 0.05$ vs. l-NAME + l-arginine), and $231.8 \pm 49.9$ s ($n = 6$), respectively.

**Fig. 2.** Representative photomicrographs of cross sections of hematoxylin-eosin-stained lymph vessels with (A) or without (B) air-rubbing treatment of endothelium. Scale bars, 50 μm. C–F: ATP-induced changes in percent frequency and cessation period of lymphatic pump activity and percent $D_{\text{max}}$ and $D_{\text{min}}$ of isolated lymph vessels with (○, $n = 7$) or without endothelium (●, $n = 7$). *Significantly different ($P < 0.05$) from intact endothelium. Percent frequency and cessation period were determined as described in Fig. 1.
Fig. 3. A: representative traces of effects of $10^{-6}$ M ATP on intrinsic lymphatic pump activity with endothelium in the absence of $N^\cdot$-nitro-L-arginine methyl ester (L-NAME, top), presence of $3 \times 10^{-5}$ M L-NAME (middle), or presence of $3 \times 10^{-5}$ M L-NAME + $10^{-3}$ M L-arginine (bottom). See Fig. 1 for an explanation of arrowheads. B–E: effects of $3 \times 10^{-5}$ M L-NAME (▲) or $3 \times 10^{-5}$ M L-NAME + $10^{-3}$ M L-arginine (■) on ATP-induced changes in percent frequency and cessation period of lymphatic pump activity and percent $D_{\text{max}}$ and $D_{\text{min}}$ of isolated lymph vessels with endothelium ($n = 6$). Percent frequency and cessation period were determined as described in Fig. 1. *Significantly different from absence of L-NAME ($P < 0.05$). †Significantly different from L-NAME alone ($P < 0.05$).
There were no significant differences in percent $D_{\text{max}}$ of the isolated lymph vessels without l-NAME, with l-NAME, or with l-NAME + l-arginine (Fig. 3D). Changes in percent $D_{\text{min}}$ mediated by $3 \times 10^{-7}$ M ATP were significantly reduced by l-NAME (Fig. 3E). l-Arginine significantly restored and exacerbated the reduction of percent $D_{\text{min}}$ mediated by $3 \times 10^{-8}$ and $10^{-7}$ M ATP (Fig. 3E).

There were no significant differences in ATP-induced inhibitory responses of the lymph vessels between the absence and presence of $10^{-5}$ M indomethacin. Thus changes in percent frequency and cessation periods of lymphatic pump activity induced by $10^{-6}$ M ATP in the presence of $10^{-5}$ M indomethacin were $25.0 \pm 7.6\%$ [$n = 6$, $P = \text{NS}$ vs. without indomethacin (26.7 ± 10.9%)] and $197.2 \pm 50.5$ s [$n = 6$, $P = \text{NS}$ vs. without indomethacin (212.7 ± 50.2 s)], respectively. There were no significant differences in percent $D_{\text{max}}$ and percent $D_{\text{min}}$ of the isolated lymph vessels between the absence and presence of indomethacin (data not shown).

Effects of glibenclamide on ATP-induced responses. In the presence of $10^{-6}$ M glibenclamide, the ATP-induced dilation with a cessation of lymphatic pump activity in the lymph vessels with endothelium was significantly reduced (Fig. 4, A and B). Changes in percent frequency of lymphatic pump activity induced by $10^{-6}$ M ATP in the absence and presence of glibenclamide were $28.4 \pm 5.3\%$ and $49.4 \pm 9.6\%$, respectively ($n = 6$, $P < 0.05$). The cessation periods of pump activity induced by $10^{-6}$ M ATP in the absence and presence of glibenclamide were $174.5 \pm 30.5$ and $103.7 \pm 37.4$ s, respectively ($n = 6$, $P < 0.05$). Thus the $10^{-6}$ M ATP-mediated dilation with a cessation of lymphatic pump activity remains in the presence of $10^{-6}$ M glibenclamide.

Effects of DPCPX, suramine, or DMPX on ATP-induced responses. In the presence of $10^{-7}$ or $3 \times 10^{-7}$ M DPCPX, the ATP-induced dilation with cessation of lymphatic pump activity in the lymph vessels with endothelium was significantly, but not completely, reduced (Fig. 5, A and B). Changes in percent frequency of lymphatic pump activity induced by $3 \times 10^{-7}$ M ATP in the absence and presence of $10^{-7}$ or $3 \times 10^{-7}$ M DPCPX were $47.6 \pm 9.4\%$ ($n = 7$) and $73.8 \pm 6.0\%$ ($n = 7$, $P < 0.05$ vs. without DPCPX) or $89.3 \pm 3.6\%$ ($n = 7$, $P < 0.05$ vs. without DPCPX), respectively. The cessation periods of lymphatic pump activity induced by $3 \times 10^{-7}$ M ATP in the absence and presence of $10^{-7}$ or $3 \times 10^{-7}$ M DPCPX were $47.6 \pm 38.3$ s ($n = 7$) and $29.9 \pm 17.9$ s ($n = 7$, $P < 0.05$ vs. without DPCPX) or $13.7 \pm 9.3$ s ($n = 7$, $P < 0.05$ vs. without DPCPX), respectively. There were no significant differences in percent $D_{\text{max}}$ of the isolated lymph vessels between the presence and absence of DPCPX (data not shown).

Pretreatment with $3 \times 10^{-7}$ M DPCPX also significantly suppressed $10^{-6}$ M ATP-mediated inhibitory responses of lymph vessels without intact endothelium (data not shown).

**Fig. 4.** Effects of $10^{-6}$ M glibenclamide (●) on ATP-induced changes in percent frequency (A) and cessation period (B) of lymphatic pump activity and percent $D_{\text{max}}$ (C) and $D_{\text{min}}$ (D) of isolated lymph vessels with endothelium ($n = 6$). Percent frequency and cessation period were determined as described in Fig. 1. *Significantly different ($P < 0.05$) from absence of glibenclamide (○).
Suramine (10^{-4} M) or 3 \times 10^{-7} M DMPX does not suppress the ATP-induced changes in percent frequency and the cessation period of lymphatic pump activity and changes in percent \(D_{\text{max}}\) and percent \(D_{\text{min}}\) of the isolated lymph vessels with endothelium (Figs. 6 and 7). Changes in percent frequency of lymphatic pump activity induced by 10^{-6} M ATP in the absence and presence of suramine were 25.3 \pm 3.6\% and 34.4 \pm 7.1\%, respectively (\(n = 5, P = \text{NS};\) Fig. 6A). The cessation periods of pump activity induced by 10^{-6} M ATP in the absence and presence of suramine were 216.6 \pm 17.3 and 213.2 \pm 11.8 s, respectively (\(n = 5, P = \text{NS};\) Fig. 6B). There were no significant differences in percent \(D_{\text{max}}\) (Fig. 6C) and percent \(D_{\text{min}}\) (Fig. 6D) of the isolated lymph vessels with endothelium between the absence and presence of suramine.

Changes in percent frequency of lymphatic pump activity in the lymph vessels with endothelium induced by 10^{-6} M ATP in the absence and presence of DMPX were 38.2 \pm 7.0\% and 32.0 \pm 6.3\%, respectively (\(n = 6, P = \text{NS};\) Fig. 7A). The cessation periods of pump activity induced by 10^{-6} M ATP in the absence and presence of DMPX were 166.5 \pm 46.6 and 154.2 \pm 45.5 s, respectively (\(n = 6, P = \text{NS};\) Fig. 7B). There were no significant differences in percent \(D_{\text{max}}\) (Fig. 7C) and percent \(D_{\text{min}}\) (Fig. 7D) of the isolated lymph vessels between the absence and presence of DMPX.

Effects of L-LAME, glibenclamide, or DPCPX on adenosine-induced responses. In some lymph vessels (24 of 30 preparations) with endothelium, 3 \times 10^{-8}–10^{-6} M adenosine (Fig. 8) caused dose-dependent dilation with a cessation of lymphatic pump activity; these results were similar to those obtained with ATP (Fig. 1A). In contrast, adenosine produced no significant dilation with a cessation of lymphatic pump activity in some lymph vessels (6 of 30 preparations). With the former lymph vessels, we examined effects of 3 \times 10^{-5} M L-NAME, 10^{-6} M glibenclamide, or 3 \times 10^{-7} M DPCPX on the adenosine-induced inhibitory responses (Fig. 9).

The decreases in percent frequency induced by 3 \times 10^{-7} M adenosine in the absence and presence of 3 \times 10^{-5} M L-NAME were 42.5 \pm 6.0\% and 75.4 \pm 6.8\%, respectively (\(n = 8, P < 0.05;\) Fig. 9A). The increases in the cessation period induced by 3 \times 10^{-7} M adenosine in the absence and presence of 3 \times 10^{-5} M L-NAME were 179.5 \pm 38.9 and 42.0 \pm 21.7 s, respectively (\(n = 8, P < 0.05;\) Fig. 9B). L-NAME caused no significant effect on the adenosine-induced increase in percent \(D_{\text{max}}\) except at 10^{-6} M adenosine (Fig. 9C). There were no significant differences in percent \(D_{\text{min}}\) (Fig. 9D) of the lymph vessels between the absence and presence of L-NAME.

The decreases in percent frequency induced by 3 \times 10^{-7} M adenosine in the absence and presence of 10^{-6} M glibenclamide were 52.7 \pm 6.8\% and 83.4 \pm 3.2\%, respectively (\(n = 8, P < 0.05;\) Fig. 9A). The increases in cessation period of pump activity induced by 3 \times 10^{-7} M adenosine in the absence and presence of glibenclamide were 146.2 \pm 41.3 and 18.0 \pm 11.4 s, respectively (\(n = 8, P < 0.05;\) Fig. 9B). There were no significant differences in percent \(D_{\text{max}}\) (Fig. 9C) of the...
lymph vessels between the absence and presence of glibenclamide. Glibenclamide significantly inhibited the adenosine (10^{-7}–10^{-6} M)-induced increase in percent $D_{\text{min}}$ (Fig. 9D).

Changes in percent frequency induced by 3 × 10^{-7} M adenosine in the absence and presence of 3 × 10^{-7} M DPCPX were 59.4 ± 8.0% and 82.5 ± 5.4%, respectively ($n = 8$, $P < 0.05$; Fig. 9A). The increase in the cessation period induced by 3 × 10^{-7} M adenosine in the absence and presence of 3 × 10^{-7} M DPCPX were 113.3 ± 39.4 and 27.5 ± 11.6 s, respectively ($n = 8$, $P < 0.05$; Fig. 9B). There were no significant differences in percent $D_{\text{max}}$ of the isolated lymph vessels between the absence and presence of DPCPX (Fig. 9C). DPCPX significantly inhibited the increase in percent $D_{\text{min}}$ induced by 10^{-7} M adenosine (Fig. 9D).

DISCUSSION

The major findings of the present study are summarized as follows. 1) ATP caused dose-dependent dilation with a cessation of intrinsic pump activity in rat isolated lymph vessels. 2) There is no tachyphylaxis. 3) The removal of lymphatic endothelium significantly reduced the ATP-mediated dilation with a cessation of lymphatic pump activity, whereas the reduction was not addressed completely with 10^{-6} M ATP. Thus the 10^{-6} M ATP-mediated inhibitory responses remain in lymph vessels without endothelium. 4) L-NAME significantly, but not completely, suppressed the ATP-induced inhibitory responses of lymphatic pump activity in lymph vessels with endothelium. Thus 10^{-6} M ATP-mediated inhibitory responses remain in the presence of L-NAME. 5) L-Arginine significantly restored the L-NAME-mediated reduction in the ATP-mediated inhibitory responses. 6) Glibenclamide significantly, but not completely, reduced the ATP-mediated inhibitory responses of lymphatic pump activity in lymph vessels with endothelium. Thus 10^{-6} M ATP-mediated inhibitory responses remain in the presence of glibenclamide. 7) DPCPX (a selective adenosine A1 antagonist), but not suramine (a P2X and P2Y receptor antagonist) or DMPX (a selective adenosine A2 antagonist), significantly suppressed, but not completely, the ATP-induced inhibitory responses in lymph vessels with or without endothelium. 8) α,β-Methylene ATP had no significant effect on lymphatic pump activity. 9) Adenosine also produced dilation with a cessation of lymphatic pump activity in some lymph vessels with endothelium (24 of 30 preparations). 10) The adenosine-mediated inhibitory responses were significantly, but not completely, reduced by treatment with L-NAME, glibenclamide, or DPCPX. Therefore, we have concluded that ATP-induced dilation and inhibition of intrinsic pump activity of isolated lymph vessels are endothelium-dependent and independent responses. Production of endogenous NO and activation of K_{ATP} channels in lymph vessels contribute, in part, to the ATP-mediated inhibitory responses of lymphatic pump activity in lymph vessels with endothelium.

Fig. 6. Effects of 10^{-4} M suramine (●) on ATP-induced changes in percent frequency (A) and cessation period (B) of lymphatic pump activity and percent $D_{\text{max}}$ (C) and $D_{\text{min}}$ (D) of isolated lymph vessels with endothelium ($n = 5$). Percent frequency and cessation period were determined as described in Fig. 1. □, Absence of suramine.

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Involvement of activation of adenosine A1 receptors in the ATP-mediated inhibitory responses. ATP-induced constrictor responses of bovine and guinea pig mesenteric lymph vessels were significantly antagonized by 10^{-4} M suramine, a blocker of P2X and P2Y receptors (7, 13, 50). The same concentration of suramine used in the present study did not antagonize the ATP-induced inhibitory responses of intrinsic pump activity in these lymph vessels of rats. In contrast, 10^{-7} and 3 \times 10^{-7} M DPCPX (a selective adenosine A1 receptor antagonist), but not 3 \times 10^{-7} M DMPX (a selective adenosine A2 receptor antagonist), significantly reduced ATP-induced inhibitory responses of lymphatic pump activity in lymph vessels with or without endothelium.

In some lymph vessels with endothelium (24 of 30 preparations), adenosine also caused dose-dependent dilation with a

Fig. 7. Effects of 3 \times 10^{-7} M 3,7-dimethyl-1-proparglyxanthine (DMPX, ●) on ATP-induced changes in percent frequency (A) and cessation period (B) of lymphatic pump activity and percent D_{max} (C) and D_{min} (D) of isolated lymph vessels with endothelium (n = 6). Percent frequency and cessation period were determined as described in Fig. 1. ○, Absence of DMPX.

Fig. 8. Representative traces of dose-dependent effects of 3 \times 10^{-8}–10^{-6} M adenosine on lymphatic intrinsic pump activity with endothelium. See Fig. 1 for an explanation of arrowheads.
cessation of lymphatic pump activity, which was quite similar to that obtained with ATP in rat iliac lymph vessels with endothelium. Thus the present study also suggests a marked heterogeneity in the adenosine-induced inhibitory responses between the lymph vessels. The adenosine-mediated inhibitory response was also significantly reduced by pretreatment with DPCPX. The concentrations of DPCPX and DMPX used in the present study were known to antagonize selectively the adenosine receptor-mediated responses in in vitro experiments (29).

The ATP-mediated inhibitory responses of the lymph vessels were significantly reduced by removal of the endothelium. In contrast, the ATP-mediated inhibitory responses of lymph vessels were slightly observed after removal of the endothelium (Fig. 2). The $10^{-6} \text{ M}$ ATP-mediated inhibitory responses of lymph vessels without endothelium were significantly reduced by pretreatment with $3 \times 10^{-7} \text{ M}$ DPCPX. These findings suggest that ATP may activate, in part, adenosine A$_1$ receptors on the endothelium and smooth muscles of the lymph vessels, resulting in dilation with a cessation of lymphatic pump activity. This conclusion may be compatible with another experimental finding that $\alpha,\beta$-methylene ATP had no significant effect on pump activity of the isolated lymph vessels with endothelium.

Adenosine receptors have been classified A$_1$, A$_2A$, A$_2B$, and A$_3$ (38). It is generally accepted that adenosine causes vasodilation via adenosine A$_2$ receptors (38). Adenosine A$_1$ and A$_2A$ receptors interact to vasodilate the renal arteriole (28). In addition, there is evidence that differential expression of adenosine receptor subtypes contributes to functional heterogeneity of endothelial cells (6). Further investigation is needed to evaluate the possibility that other adenosine receptors may be involved in ATP-mediated inhibitory responses of lymphatic pump activity. In addition, we should study in the future why adenosine has no significant effect on lymphatic pump activity in some lymph vessels.

**Involvement of NO and K$_{ATP}$ channels in the ATP-mediated inhibitory responses.** NO has an important role in control of physiological functions in the lymph vessels. Thus NO causes relaxation of lymphatic smooth muscles and inhibits intrinsic pump activity of lymph vessels in vivo and in vitro studies (24, 26, 27, 35, 40, 45, 48). Recently, it has also become clear that NO has a significant role in flow-mediated dilation with a cessation of lymph pump activity (8, 40, 42). K$_{ATP}$ channels also significantly contribute to regulation of intrinsic pump activity in lymph vessels (25–27, 44). In the present study, ATP produced dilation with a cessation of lymphatic pump activity in lymph vessels with or without endothelium. The ATP-mediated inhibitory responses were significantly, but not completely, reduced by pretreatment with L-NAME (an NO synthase inhibitor) or glibenclamide (a K$_{ATP}$ channel blocker), but not indomethacin (a cyclooxygenase inhibitor). In addition, in some preparations with endothelium, adenosine-mediated inhibitory responses were significantly, but not completely, reduced by pretreatment with L-NAME or glibenclamide. These findings suggest that the ATP- or adenosine-mediated inhibitory responses of lymphatic pump activity are involved, in part, in the production of NO and activation of K$_{ATP}$ channels. The findings are quite compatible with the conclusion that adenosine dilated rat diaphragmatic arterioles with involvement of NO production and activation of K$_{ATP}$ channels (4).

In the present study, l-arginine significantly restored and also exacerbated the ATP-mediated inhibitory responses in lymph vessels with endothelium (Fig. 3). Extracellular l-arginine is required for optimal NO synthesis by endothelial NO synthase in rat mesenteric artery (20). The concentration of l-arginine may affect the ATP-mediated NO synthase. Thus further investigation is needed to study the effect of l-arginine on the ATP-mediated NO production and/or activity in lymph vessels. However, $10^{-6} \text{ M}$ ATP- or adenosine-mediated inhibitory responses remain in the presence of $3 \times 10^{-5} \text{ M}$ L-NAME, $10^{-6} \text{ M}$ glibenclamide, or $3 \times 10^{-7} \text{ M}$ DPCPX. Further investigation is needed to evaluate the other mechanisms involved in the inhibitory responses mediated by $10^{-6} \text{ M}$ ATP or adenosine.

**ATP-mediated inhibitory responses of lymphatic pump activity.** It is well known that myocytes in the heart produce ATP, which is rapidly degraded to ADP, AMP, and adenosine by ectoenzymes. The myocyte-derived adenosine is a key substance in autoregulation of the coronary arterial system (11, 12, 19, 43). Adenosine may play similar crucial roles in the autoregulatory function of the aortic (5), cerebral (14), and renal vasculature (17). Recently, it was reported that cultured pulmonary arterial endothelial cells endogenously produce ATP in response to shear stress and that endogenous release of ATP plays a significant role in the regulation of functions of cultured endothelial cells (47). Immunohistochemical studies indicate the presence of 5’-nucleotidase, an ectoenzyme that converts adenine nucleotides to adenosine, in endothelial cells of lymphatic capillaries and lymph vessels (18, 46, 51). However, a study of metabolism of adenosine nucleotides in the lymphatic wall has not been reported.

Lymph vessels exhibit a marked intrinsic contraction and relaxation, which serve as an active pump to transport lymph in vivo and in vitro. ATP increases the frequency of intrinsic pump activity of mesenteric lymph vessels in sheep (13) and guinea pigs (7, 50). The ATP-mediated constritor response is independent of the function of the endothelium in mesenteric lymph vessels of guinea pigs (50). In the present study, ATP dilated rat iliac lymph vessels and inhibited the frequency of intrinsic pump activity of these vessels. $\alpha,\beta$-Methylene ATP, a selective P2X and P2Y receptor agonist for lymph vessels (50), did not affect frequency and amplitude of pump activity of isolated rat afferent lymph vessels. In addition, the ATP-mediated inhibition of rat isolated lymph vessels is an endothelium-dependent and -independent response, in marked contrast to the above-mentioned ATP-mediated excitatory responses of sheep and guinea pig lymph vessels. These findings may indicate a marked heterogeneity, including species and regional differences, receptor subtypes, and intracellular signal transduction mechanisms, in ATP-mediated responses of internal...
trisinic pump activity of lymph vessels. Further investigation is needed to analyze the physiological, pharmacological, and morphological characteristics of the heterogeneity in ATP-mediated responses in the lymph vessels.

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REFERENCES


