Glucose and Glucose Transporters Regulate Lymphatic Pump Activity through Activation of the Mitochondrial ATP-Sensitive K+ Channel

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Abstract: We investigated the pivotal roles of glucose and its transporter in the regulation of mechanical activity of isolated rat thoracic ducts and then examined whether mitochondrial ATP-sensitive K+ channels (mitoK_ATP) are involved in those responses. In the absence of extracellular glucose, the thoracic ducts showed pump activity during 120 min. Extracellular glucose caused a dose-dependent increase in the frequency of pump activity and a constriction in the thoracic ducts. Pump activity of the thoracic ducts in 0 mM glucose was completely inhibited in the presence of chlorogenic acid (an inhibitor of glucose-6-phosphatase). Cytochalasin B, an inhibitor of facilitative glucose transporter (GLUT), or phlorizin, an inhibitor of sodium-dependent glucose cotransporter (SGLT), significantly reduced the frequency of pump activity and dilated the thoracic ducts. A decrease in the frequency of pump activity induced by 5-hydroxydecanoate (5-HD, a selective blocker of mitoK_ATP) was completely reversed by ruthenium red (an inhibitor of Ca2+-uniporter in mitochondria). Diazoxide (a selective opener of mitoK_ATP) significantly increased the frequency of pump activity. Carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP, a protonophore of mitochondrial proton pump action) significantly reduced the frequency of pump activity and dilated the thoracic ducts. Collectively, these findings suggest that glucose derived from intracellular glycogen and/or through GLUT/SGLT in lymphatic smooth muscles contributes to the regulation of the pump activity of isolated rat thoracic ducts, and that mitoK_ATP in the cells may partially serve as a modulator of the mechanical functions associated with mitochondrial Ca2+ uptake.

Key words: lymph vessel, smooth muscle, transporter, ATP-sensitive K+ channel.

The lymphatic system utilizes an important method of microcirculation to regulate the transport of extracellular fluids and macromolecular substances in tissues. Lymph vessels act to return fluids and protein, especially albumin, that escape from the capillary to the interstitial space [1]. It is clear that lymph vessels work as a series of lymphatic pumps to propel the lymph fluid centripetally by rhythmic constriction and dilation. Smooth muscle cells in the vessels of humans and animals have unique characteristics that exhibit spontaneous activity of the cells, which serve as the main machinery of the pump in vivo and ex vivo [2]. It is well known that spontaneous activity of gastrointestinal smooth muscle cells is considered to be produced in interstitial cells of Cajal (ICC) or ICC-like cells. The role of mitochondria in the rhythmic activity of cells, which is developed in relation to the Ca2+-handling mechanism in mitochondria, has been demonstrated in gastrointestinal pacemaker cells [3–5]. Morphological studies also suggest that there are c-kit positive ICC-like cells in the lymph vessel walls of sheep [6].

Morphological studies of lymphatic smooth muscle cells have demonstrated the existence of numerous mitochondria and glycogen granules in the cells [7]. Moreover, glycogen granules are located near the mitochondria. The presence of mitochondria and glycogen granules seems to produce ATP in the lymphatic smooth muscles with spontaneous activity. These morphological findings may be associated with findings that lymphatic smooth muscle cells have ATP-sensitive K+ channels (K_ATP), which contribute to the regulation of functions including the formation of membrane potentials, maintenance of myogenic tone, and contractility and/or frequency of lymph pump activity [8–13]. We have shown that the mechanical activities of lymphatic smooth muscles are governed by metabolic dependent regulatory mechanisms, such as the intracellular concentration of ATP. It is known that K_ATP first found in cardiac muscle [14], is widely located on the plasma membrane in smooth muscle cells of various organs [15, 16]. Several pharmacological studies, however, have demonstrated that another K_ATP mitochondrial K_ATP (mitoK_ATP), in cardiac muscle cells plays significant roles in pathophysiological conditions, such as ischemia [17–21]. Few reports are available regarding the involvement
of mitoK$\text{ATP}$ in the regulation of physiological functions of smooth muscle cells in lymph vessels.

Glucose is one of the sources for producing intracellular ATP in mammalian cells. In accordance with the demand for glucose, mammalian cells are equipped with glucose transporters, which consist of a facilitative glucose transporter (GLUT) and a sodium-dependent glucose cotransporter (SGLT) [22]. GLUT allows the energy-independent and concentration gradient–dependent transport of glucose across the hydrophobic cell membrane and exists in all cells. SGLT is a secondary active transport of glucose across the hydrophobic cell membrane and exists in all cells. GLUT and SGLT are distributed to smooth muscle cells of blood vessels and that they participate in the regulation of vascular functions [23–25].

PO$_2$ in the lymphatic circulation is known to be 25–40 mmHg, lower than that obtained through blood circulation in normal physiological conditions [2, 26, 27] and indicating that the lymphatic smooth muscle cells modulate lymph pump activity under low oxygen tension. Collectively, the physiology of lymphatic smooth muscle cells can be summarized as follows: (1) the cells cells show a marked spontaneous contractility; (2) for contractile energy, the cells produce ATP by using an extracellular glucose; (3) for the regulation of activity, the cells activate/inactivate K$\text{ATP}$ located on the plasma membrane; (4) all events operate under low oxygen tension. Here, several questions arise why lymphatic smooth muscle cells can function under low oxygen conditions, which glucose transporters of the cells are involved in the regulation, and how mitoK$\text{ATP}$ is associated with cellular functions. It is important to elucidate these matters to provide new insights for understanding the physiological significance of the lymphatic system. Therefore in the present study we have first examined the effects of glucose and glucose transporters (GLUT and SGLT) on the mechanical activity of smooth muscles in lymph vessels. We then studied the involvement of mitoK$\text{ATP}$ in the regulation of lymphatic mechanical activity by using isolated rat thoracic duct preparations.

**MATERIALS AND METHODS**

**Animals.** Seven- to 8-week-old male Wistar rats ($n = 55$; 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. The rats were anesthetized with pentobarbital sodium (50 mg/kg ip) and exsanguinated. This drug was widely used for experimental rats and was 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. After an incision of the thoracic cavity, the thoracic ducts were excised and placed in a petri dish containing cold (4°C) Krebs-bicarbonate solution (KBS, in mM: 120.0 NaCl, 5.9 KCl, 2.5 CaCl$_2$, 1.2 MgSO$_4$, 1.2 NaH$_2$PO$_4$, 5 glucose, and 25.0 NaHCO$_3$). In the present study, we used KBS containing different concentrations of glucose (0, 0.1, 0.5, 1, 3, and 5 mM), prepared by replacing glucose with equimolar amounts of mannitol [28]. With microsurgical instruments and an operating microscope, the thoracic ducts ($n = 55$, maximum diameter of 340–657 µm, and ~3 mm long) were isolated and then transferred to a 10-ml organ chamber with two glass micropipettes containing KBS with 0 mM glucose. After each thoracic duct was mounted on a pipette (proximal) and secured with sutures, the perfusion pressure was raised to 4 cmH$_2$O to flush out and clear the vessel. Subsequently, the distal end of the thoracic ducts was mounted on the outflow micropipette (distal). The proximal and distal micropipettes were connected through Tygon tubing with a 25-ml syringe and a stopcock, respectively. Intraluminal space of the thoracic ducts, tubing, and the pressure column were filled with 0 mM glucose KBS. KBS (glucose concentration: 0, 0.1, 0.5, 1, 3, or 5 mM), by bubbling with 5% CO$_2$–95% N$_2$, was perfused extraluminally over the thoracic ducts within the organ chamber. Previously we have measured PO$_2$ values (40–50 mmHg) in KBS, thus KBS with 5% CO$_2$–95% N$_2$ is useful for studying lymphatic circulation in vivo and ex vivo [9–12, 29–31]. In fact, PO$_2$ in the lymphatic circulation is known to be 25–40 mmHg, which is lower than that obtained through blood circulation under physiological conditions [2, 26, 27]. The flow rate of the superfused solution was kept at 5 ml/min throughout the experiment. After cannulation of the thoracic ducts, the chamber was transferred to the stage of a microscope (Nikon Eclipse E600). The thoracic ducts were then warmed slowly to 37°C and allowed to equilibrate for ~40 min.

**Measurement of lymph vessel diameter.** An objective lens (×2) and a monochrome charge-coupled device camera (KOKOM KCB-270A) were used to obtain images of the thoracic ducts, which were displayed on a monochrome television monitor (Hamamatsu Photonics). The diameter of the thoracic ducts was manually and automatically measured with a diameter-detection device with an edge-detection method [30]. They were recorded on a videocassette recorder (Toshiba) and a direct-writing oscillograph (Sanei-Sokki, Recti 8K). Intraluminal pressure in the thoracic ducts was kept at 7 cmH$_2$O by the ele-
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vation of a 25-ml syringe connected to the inflow tubing; the outflow tubing was closed with a stopcock throughout the experiments. This pressure was optimal for producing pump activity in the isolated Wistar rat thoracic ducts.

Experimental protocol. In the first experiment, we examined the time-dependent effects of the superfusion of 0 mM glucose on the mechanical activity of thoracic ducts. Following equilibrium with 5 mM glucose for 60 min, 0 mM glucose was superfused extraluminally over the thoracic ducts for 120 min. In this protocol we recorded changes in the diameter of the ducts for 120 min and measured the diameters and frequency before (at 0 min) and in 30 min (at 30, 60, 90, and 120 min) intervals. Moreover, we studied the effect of the superfusion of 0 mM glucose on the activity of thoracic ducts in the presence of an inhibitor of glucose-6-phosphatase, chlorogenic acid (10⁻⁴ M) that terminates intracellular glucose production from glycogen [32]. We next examined the concentration-dependent effects of glucose (0, 0.1, 0.5, 1, 3, and 5 mM) on the activity of thoracic ducts. Each concentration of glucose was superfused extraluminally over the thoracic ducts until stable activity (~30 min) was obtained. Our preliminary experiments revealed no significant differences in the mechanical parameters of lymphatic pump activity from 3 mM to 5 mM glucose. Thus we accepted that 3 mM glucose was the maximum dose for investigation in the present study. We set the three concentrations of glucose (0.1 mM, 1 mM, or 3 mM) from quite low to sufficiently high for use in the subsequent experiments.

In the second experiment, we examined the effects of cytochalasin B (an inhibitor of GLUT; 10⁻⁶ M) [33] or phlorizin (an inhibitor of SGLT; 3 x 10⁻⁴ M) [23] on pump activity of the thoracic ducts in 0.1, 1, or 3 mM glucose.

In the third experiment, we examined the effects of 5-hydroxydecanoate (5-HD, a selective mitochondrial K_ATP blocker; 3 x 10⁻³ M) [18] on pump activity of the thoracic ducts in 0.1, 1, or 3 mM glucose. 5-HD-mediated responses of the thoracic ducts were also investigated in the presence of ruthenium red (a selective inhibitor of mitochondrial Ca²⁺ uniporter; 2 x 10⁻⁷ M) [34]. Further, we examined the effects of diazoxide (a selective mitochondrial K_ATP opener in cardiac muscles and vascular smooth muscles; 10⁻³ M) [18, 35, 36] on pump activity of the thoracic ducts in 0.1, 1, or 3 mM glucose.

In the final experiment, we examined the effects of carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP, a protonophore; 10⁻⁷ M) [19] on pump activity of the thoracic ducts in 0.1, 1, or 3 mM glucose. This protonophore prevents the maintenance of mitochondrial membrane potential at negative level.

Drugs. All salts were obtained from Wako; mannitol, phlorizin, cytochalasin B, 5-HD, ruthenium red, diazoxide, and FCCP from Sigma; chlorogenic acid from MP Biomedicals. Cytochalasin B was diluted with DMSO. Diazoxide and FCCP were diluted with methanol and ethanol, respectively. The concentrations of DMSO, methanol, and ethanol did not affect the myogenic tone and pump activity of the isolated thoracic ducts. Concentrations of drugs were expressed as the final concentrations in the organ chamber. All salts and drugs were prepared on the day of the experiment.

Statistical analyses. The frequency (min⁻¹) of pump activity and maximum diameter (D_max, µm) and minimum diameter (D_min, µm) of the thoracic ducts were measured. The amplitude (D_max – D_min, µm) of lymphatic pump activity was also calculated. Changes in the parameters during the glucose- or drug-induced responses were normalized by each control value of the parameters obtained before application of the drugs or glucose [9–12, 29]. Values are means ± SE, and n indicates the number of vessels. Significant differences (p < 0.05) were determined by one-way ANOVA followed by a Student-Newman-Keuls or Dunnett post hoc test and unpaired and paired Student’s t-test, as appropriate.

RESULTS

Time-dependent effects of 0 mM glucose on pump activity of isolated thoracic ducts

In 5 mM glucose, the isolated thoracic duct exhibited stable spontaneous activity (Fig. 1A, left). The thoracic duct still demonstrated this activity even after the superfusion of 0 mM glucose for 120 min (Fig. 1A, center and right). The frequency of pump activity significantly and time-dependently decreased after the superfusion of 0 mM glucose. The frequencies before (at 0 min) and after the superfusion of 0 mM glucose (at 120 min) were 16 ± 1 min⁻¹ and 4 ± 0 min⁻¹ (n = 10, p < 0.05 vs. at 0 min), respectively (Fig. 1B). After the superfusion of 0 mM glucose, the D_max and D_min of the thoracic ducts significantly and time-dependently increased (Fig. 1A, center and right). The D_max of the thoracic ducts before (at 0 min) and after the superfusion (at 120 min) of 0 mM glucose were 494 ± 22 µm (n = 10) and 540 ± 16 µm (n = 10, p < 0.05 vs. at 0 min), respectively (Fig. 1C, open circles). The D_min of the thoracic ducts before (at 0 min) and after the superfusion (at 120 min) of 0 mM glucose were 371 ± 23 µm (n = 10) and 429 ± 24 µm (n = 10, p < 0.05 vs. at 0 min), respectively (Fig. 1C, closed circles). Values for the amplitude of pump activity were not significantly different before (at 0 min, 123 ± 11 µm, n = 10) and after (at 120 min, 111 ± 12 µm, n = 10) the superfusion of 0 mM glucose (Fig. 1D).

Dose-dependent effects of glucose on pump activity of isolated thoracic ducts

An increase in the concentration of glucose significantly and dose-dependently increased the frequency of pump activity (Fig. 2A). The value of the frequency reached a plateau at 3 mM glucose. The frequencies in
0 mM and 3 mM glucose were 4 ± 1 min⁻¹ (n = 6, p < 0.05 vs. 3 mM glucose KBS) and 17 ± 1 min⁻¹ (n = 6), respectively (Fig. 2B). An increase in the concentration of glucose significantly and dose-dependently constricted the thoracic ducts. The values of Dₘₐₙ and Dₘᵦᵣ reached a plateau at 3 mM glucose. The Dₘₐₓ of the thoracic ducts in 0 mM and 3 mM glucose were 543 ± 27 µm (n = 6, p < 0.05 vs. 3 mM glucose) and 425 ± 31 µm (n = 6), respectively (Fig. 2C, open circles). The Dₘᵦᵣ of the thoracic ducts in 0 mM and 3 mM glucose were 441 ± 41 µm (n = 6, p < 0.05 vs. 3 mM glucose) and 320 ± 36 µm (n = 6), respectively (Fig. 2C, closed circles). The values of amplitude of pump
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The effects of chlorogenic acid (10^{-4} M) on the diameter of an isolated rat thoracic duct in 5 mM or 0 mM glucose. Percent frequency (B), percent D_{max} (C), percent D_{min} (D), and percent amplitude (E) of pump activity of isolated thoracic ducts before (at 0 min) and after the superfusion of 0 mM glucose in the absence (open circles, n = 4) or presence (closed circles, n = 4) of chlorogenic acid (CA, 10^{-4} M). * indicates significant difference (p < 0.05) from the absence of chlorogenic acid.

Effects of chlorogenic acid on pump activity of isolated thoracic ducts in 0 mM glucose

In the presence of 5 mM glucose, chlorogenic acid did not affect pump activity of the thoracic duct (Fig. 3A). In the presence of 10^{-4} M chlorogenic acid, the frequency of pump activity time-dependently decreased following the superfusion of 0 mM glucose and reached 0 after the superfusion at 90–120 min (Fig. 3A). The percent frequencies after the superfusion of 0 mM glucose at 120 min in the absence and presence of 10^{-4} M chlorogenic acid were 26 ± 2% (n = 4) and 0 ± 0% (n = 4, p < 0.05 vs. absence), respectively (Fig. 3B). In the presence of 10^{-4} M chlorogenic acid, the isolated thoracic duct showed a marked dilation after the superfusion of 0 mM glucose (Fig. 3A). The percent D_{max} of the thoracic ducts following the superfusion of 0 mM glucose at 120 min in the presence of 10^{-4} M chlorogenic acid (114 ± 5%, n = 4, p < 0.05 vs. absence) was significantly larger than that obtained in its absence (105 ± 0%, n = 4) (Fig. 3C). The percent D_{min} of the thoracic ducts after the superfusion of 0 mM glucose at 120 min in the presence of 10^{-4} M chlorogenic acid (149 ± 17%, n = 4, p < 0.05 vs. absence) was significantly larger than that obtained in its absence (108 ± 3%, n = 4) (Fig. 3D). In the presence of 10^{-4} M chlorogenic acid, the percent amplitude of pump activity reached 0 after the superfusion of 0 mM glucose at 90–120 min, because oscillation of the diameter of the vessels disappeared completely (Fig. 3, A and E).

Effects of cytochalasin B or phlorizin on pump activity of isolated thoracic ducts

A higher concentration of cytochalasin B or phlorizin caused an irreversible effect on pump activity of the thoracic ducts or disrupted stable constriction/dilation of the vessels. Therefore we used the lower and effective concentrations of those inhibitors in the present study. We found that 10^{-6} M cytochalasin B reduced the frequency of pump activity in 0.1 mM (Fig. 4A, left) and 3 mM glucose (Fig. 4A, right). The percent frequency of pump activity after treatment of 10^{-6} M cytochalasin B in 0.1 mM and 3 mM glucose were 38 ± 4% (n = 6, p < 0.05 and 0.01 vs. absence), respectively (Fig. 4A, left and right).
vs. before) and 70 ± 5% (n = 6, p < 0.05 vs. before), respectively (Fig. 4B). There were significant differences of cytochalasin B–induced reduction of percent frequency between 0.1, 1, and 3 mM glucose (Fig. 4B). Significant changes were caused by 10\(^{-6}\) M cytochalasin B in percent D\(_{\text{max}}\) (Fig. 4C), percent D\(_{\text{min}}\) (Fig. 4D), or percent amplitude (Fig. 4E) of pump activity. In 1 mM glucose, percent D\(_{\text{max}}\), percent D\(_{\text{min}}\) and percent amplitude after treatment of 10\(^{-6}\) M cytochalasin B were 104 ± 1% (n = 6, p < 0.05 vs. before), 112 ± 5% (n = 6, p < 0.05 vs. before) and 73 ± 12% (n = 6, ns vs. before), respectively.

The frequency of pump activity in 0.1 mM glucose was reduced by 3 \times 10^{-4} M phlorizin (Fig. 5A, left), but it was not affected while in the presence of 3 mM glucose 3 \times 10^{-4} M phlorizin (Fig. 5A, right). The percent frequency of pump activity after treatment of 3 \times 10^{-4} M phlorizin in 0.1 mM and 3 mM glucose were 62 ± 4% (n = 5, p < 0.05 vs. before) and 103 ± 5% (n = 5, ns vs. before), respectively (Fig. 5B). There were significant differences in phlorizin-induced reduction of percent frequency from 0.1, 1, and 3 mM glucose (Fig. 5B). We noted that 3 \times 10^{-4} M phlorizin caused significant changes in percent D\(_{\text{min}}\) (Fig. 5D) of the thoracic ducts. In 1 mM glucose, percent D\(_{\text{max}}\), percent D\(_{\text{min}}\) of the thoracic ducts, and percent amplitude of pump activity after treatment of 3 \times 10^{-4} M phlorizin were 105 ± 2% (n = 5, ns vs. before), 109 ± 3% (n = 5, p < 0.05 vs. before), and 90 ± 8% (n = 5, ns vs. before), respectively.

**Effects of ruthenium red on 5-HD–induced responses of isolated thoracic ducts**

No effects from 2 \times 10^{-7} M ruthenium red itself were observed on the diameter of isolated rat thoracic ducts in 0.1 mM, 1 mM, or 3 mM glucose. A higher concentration of 5-HD caused an irreversible effect on pump activity of the thoracic ducts or disrupted stable constriction/dilation of the vessels. Therefore we used the lower and effective concentrations of 5-HD in the present study.

The frequency of pump activity was reduced by 3 \times 10^{-5} M 5-HD in 0.1 mM glucose (Fig. 6A, left). In the presence of 2 \times 10^{-7} M ruthenium red, the 3 \times 10^{-5} M 5-HD caused no reduction of the frequency of pump activity (Fig. 6A, right). These results are summarized in Fig. 6, B–E. In the absence of 2 \times 10^{-7} M ruthenium red, percent frequency after the treatment of 3 \times 10^{-5} M 5-HD (79 ± 2%, n = 5, p < 0.05 vs. before) in 1 mM glucose was...
Fig. 5. A: Representative tracings of the effects of phlorizin ($3 \times 10^{-4}$ M) on the diameter of an isolated rat thoracic duct in 0.1 mM (left) or 3 mM (right) glucose. Percent frequency (B), percent $D_{\text{max}}$ (C), percent $D_{\text{min}}$ (D), and percent amplitude (E) of pump activity of isolated thoracic ducts after the application of phlorizin ($3 \times 10^{-4}$ M) in 0.1, 1, or 3 mM glucose ($n = 5$). * indicates significant difference ($p < 0.05$) from before application of phlorizin. † indicates significant difference ($p < 0.05$) from 0.1, 1, and 3 mM glucose.

Fig. 6. A: Representative tracings of the effects of ruthenium red (RR, $2 \times 10^{-7}$ M) on $3 \times 10^{-5}$ M 5-HD-mediated responses of the diameter of isolated rat thoracic ducts in 0.1 mM glucose. Percent frequency (B), percent $D_{\text{max}}$ (C), percent $D_{\text{min}}$ (D), and percent amplitude (E) of pump activity of isolated thoracic ducts after the application of 5-HD ($3 \times 10^{-5}$ M) in 0.1, 1, or 3 mM glucose in the absence (open columns, $n = 5$) and presence (closed columns, $n = 5$) of ruthenium red. * indicates significant difference ($p < 0.05$) from before application of 5-HD. † indicates significant difference ($p < 0.05$) from 1 to 3 mM glucose. # indicates significant difference ($p < 0.05$) from the absence of ruthenium red.
significantly less than that obtained before (Fig. 6B, open columns). There were significant differences in 5-HD–induced reduction of the percent frequency of pump activity from 1 mM to 3 mM glucose in the absence of ruthenium red (Fig. 6B, open columns). In 1 mM glucose, $3 \times 10^{-5}$ M 5-HD–mediated percent frequency in the absence and presence of $2 \times 10^{-7}$ M ruthenium red were $79 \pm 2\%$ ($n = 5$) and $100 \pm 3\%$ ($n = 5$, $p < 0.05$ vs. absence), respectively, indicating that ruthenium red significantly and completely reversed 5-HD–mediated reduction of the frequency of pump activity (Fig. 6B, closed columns). On the other hand, $3 \times 10^{-5}$ M 5-HD caused no significant changes in percent $D_{\text{max}}$ (Fig. 6C) or percent $D_{\text{min}}$ (Fig. 6D) of the thoracic ducts, or in percent amplitude (Fig. 6E) of pump activity in each concentration of glucose KBS, examined in the absence or presence of $2 \times 10^{-7}$ M ruthenium red.

**Effects of diazoxide on pump activity of isolated thoracic ducts**

In our preliminary experiments, a higher concentration of diazoxide either caused an irreversible effect on pump activity of the thoracic ducts or disrupted stable constriction/dilation of the vessels. Therefore we used the lower and effective concentrations of diazoxide in the present study. The frequency of pump activity was increased by $10^{-7}$ M diazoxide in 1 mM glucose (Fig. 7A), and $10^{-7}$ M diazoxide caused slight and significant increases in percent frequency in 1 mM and 3 mM glucose. In 1 mM glucose, percent frequency of pump activity after $10^{-7}$ M diazoxide was $109 \pm 2\%$ ($n = 5$, $p < 0.05$ vs. before) (Fig. 7B). On the other hand, $10^{-7}$ M diazoxide caused no significant effects on percent $D_{\text{max}}$ or percent $D_{\text{min}}$ of the thoracic ducts in each concentration of glucose KBS. In 1 mM glucose, percent $D_{\text{max}}$ (Fig. 7C) and percent $D_{\text{min}}$ (Fig. 7D) of the thoracic ducts after treatment of $10^{-7}$ M diazoxide were $99 \pm 1\%$ ($n = 5$, ns vs. before) and $102 \pm 2\%$ ($n = 5$, ns vs. before), respectively.

**Effects of FCCP on pump activity of isolated thoracic ducts**

We found that $10^{-7}$ M FCCP reduced the frequency of...
pump activity in 3 mM glucose (Fig. 8A). The percent frequency after treatment of $10^{-7}$ M FCCP (80 ± 5% in 1 mM glucose, $n = 5$, $p < 0.05$ vs. before) was significantly less than that obtained before (Fig. 8B). Slight or significant changes were caused by $10^{-7}$ M FCCP in percent $D_{\text{max}}$ (Fig. 8C) and percent $D_{\text{min}}$ (Fig. 8D) of the thoracic ducts or on percent amplitude (Fig. 8E) of pump activity. In 1 mM glucose, percent $D_{\text{max}}$, percent $D_{\text{min}}$, and percent amplitude after treatment of $10^{-7}$ M FCCP were 103 ± 2% ($n = 5$, ns vs. before), 110 ± 3% ($n = 5$, $p < 0.05$ vs. before), and 72 ± 8% ($n = 5$, $p < 0.05$ vs. before), respectively.

**DISCUSSION**

**Involvement of intra- and extracellular glucose in the regulation of lymphatic pump activity**

In the present study, isolated rat thoracic ducts at a constant perfusion pressure of 7 cmH$_2$O (no-flow condition) showed stable constriction/dilation under different concentrations of extracellular glucose (0–5 mM). We find it of interest that the thoracic ducts can maintain pump activity for up to 120 min in 0 mM glucose. The superfusion of 0 mM glucose dilated the vessels and decreased the frequency of pump activity time-dependently. At 120 min after the superfusion of 0 mM glucose, the vessels kept a beat of 4 times/min. Moreover, studies of the dose-dependent effects of glucose on the thoracic ducts indicate that mechanical activity reaches a plateau at 3 mM glucose. The glucose concentration in serum is restrictively controlled at ~5 mM. The lymph/serum ratio is ~1.1, indicating that the glucose concentration of lymph is higher than that obtained with serum in whatever species it has been measured [37]. This ratio is thought to be due to concentrating mechanisms of the lymph vessels [31, 38] and lymph nodes [39, 40]. Our current in vitro studies together with those measurements suggest that lymphatic function can operate under lower glucose concentrations than is normal physiologically, and that glucose concentration under physiological conditions seems to be sufficient to maintain mechanical activity in the thoracic ducts.

It is well known that lymphatic smooth muscle cells
Fig. 9. A schema suggested by the present study, including the glucose-ATP production pathway, location of transporters/K\textsubscript{ATP}, and the action sites of drugs in lymphatic smooth muscle cells and mitochondria. FRE, frequency; G6Pase, glucose-6-phosphatase; EMP, Embden-Meyerhof-Parnas pathway; ETC, electron transport chain; UP, mitochondrial Ca\textsuperscript{2+} uniporter; [Ca\textsuperscript{2+}], intracellular concentration of Ca\textsuperscript{2+}; P, protonophore; \(\Delta\psi\), \(\Delta\) membrane potential. Boxes A, B, and C show studies of 5-HD with/without ruthenium red, diazoxide, and FCCP, respectively. Dashed arrows indicate the inhibitory actions.

mainly produce forces for the mechanical activity of lymph vessels [1, 2]. Thus glucose-dependent action in the present study originated from the mechanical activity of smooth muscle cells in the walls of the thoracic ducts. In the present study, we showed that chlorogenic acid (an inhibitor of 6-glucose-phosphatase, which inhibits the glucose production pathway from glycogen) [32] completely eliminated pump activity of the thoracic ducts observed in 0 mM glucose. The 10\textsuperscript{-4} M chlorogenic acid used in the present study was 1/10 that of the other study [32], and the concentration was effective enough to inhibit pump activity of the thoracic ducts in the absence of extracellular glucose. We speculate that the effectiveness of the low concentration of chlorogenic acid may be due to the absence of extracellular glucose. These findings suggest that intracellular glucose derived from glycogen in lymphatic smooth muscle cells may contribute, in part, to the appearance of pump activity in the absence of extracellular glucose (Fig. 9). To investigate physiological function in the lymphatic system in vivo and ex vivo experiments, we and others used a physiological salt solution containing 5–5.5 mM glucose [9–12, 29–31, 41, 42], and higher concentrations of sugars (11 mM) were also acceptable [43–45]. This study is the first demonstration that lymphatic smooth muscle cells of the thoracic ducts may merely use glucose and that glycogen-derived intracellular glucose may be utilized under a low-oxygen condition. The lymphatic system is the only route to recirculate extravasated fluids and plasma protein that cannot directly return to the blood system under a low-oxygen environment. We propose here that the lymphatic system may collect sugars to minimize the loss during lymph transport. In other words, the lymph vessels do not need a plasma level of glucose relating to the lymphatic low-oxygen system.

Roles of GLUT and SGLT in the regulation of lymphatic pump activity

Kaiser \textit{et al.} reported that cytochalasin B (10\textsuperscript{-5} M) inhibited ~90% of hexose (2-deoxy-D-glucose, 0.1 mM) uptake into cultured vascular smooth muscle cells, whereas phlorizin (5 \times 10\textsuperscript{-5} M) showed ~20% inhibitory action [23]. Quinn \textit{et al.} (1998) used 1.5 \times 10\textsuperscript{-5} \textmu M cytochalasin B to induce the complete inhibition of nonspecific GLUT [25]. Also, 10\textsuperscript{-3} M phlorizin inhibited insulin-induced glucose uptake into cultured vascular smooth muscle cells [46]. The low concentration of cytochalasin B (10\textsuperscript{-6} M) used in the present study was effective in inhibiting pump activity of the thoracic ducts. We found that 3 \times 10\textsuperscript{-4} M phlorizin used in the present study was within the effective concentration for inhibiting SGLT. In our preliminary experiments, a higher concentration of cytochalasin B or phlorizin caused an irreversible effect on pump activity of the thoracic ducts or disrupted stable
constriction/dilation of the vessels. Therefore the concentra-
tions of those inhibitors were accepted as effective for
the present study. Phlorizin reduced pump activity of the
thoracic ducts in the presence of 0.1 mM or 1 mM, but not
in 3 mM, glucose. Cytochalasin B reduced pump activity
of the thoracic ducts at all concentrations of glucose used.
Thus it is possible that GLUT and SGLT work at low-high
and low extracellular concentrations of glucose, respective-
lively. Collectively, the findings from the present study
suggest that glucose via GLUT and SGLT located on the
plasma membrane of lymphatic smooth muscle cells may
contribute, in part, to the regulation of the mechanical
activity of the lymph vessels (Fig. 9). In the present tissue
studies, used concentrations of GLUT or SGLT inhibi-
tion have been limited because of their side effects. Thus
other analyses such as a biochemical approach by using
cultured lymphatic cells will support our current investi-
gations.

More than 13 members of the mammalian GLUT
family have been identified [22, 47]. GLUTs are further
divided into three Classes (I, II, and III). The class I fa-
cilitative transporters contain GLUT1–4 that have been
comprehensively characterized in terms of structure,
function, and tissue distribution. In class I, GLUT4 is in-
sulin-sensitive transport glucose that is mainly located
on hearts, skeletal muscles, adipose (white and brown)
tissues, and brain. The present study could not identify
types of GLUT because of the nonselectivity of cytocha-
lasin B. However, the results of the present study suggest
that insulin-insensitive glucose transport mechanisms
may be involved in cytochalasin B–mediated responses
of isolated thoracic ducts, because the physiological salt
solution used in the present study contained no insulin,
and we rinsed the vessels for more than 120 min with the
solution during the equilibration period and before onset
of the protocols. It has been reported that cytochalasin B
caus ed depolymerization of F-actin that inhibited the de-
velopment of myogenic tone and decreased the effective-
ness of myogenic reactivity of cerebral arteries [48]. No
significant dilation of the cerebral arteries was caused by
10^{-6} M cytochalasin B, whereas inhibition of myogenic
activity in the vessels was observed at more than 3 \times 10^{-6}
M cytochalasin B [48]. The effective concentration of
cytochalasin B for reducing pump activity of the thoracic
ducts was 10^{-6} M in the present study. The present study
also demonstrated that cytochalasin B–mediated inhibi-
tory responses were strongly dependent on extracellular
glucose concentration, suggesting that cytochalasin B
may inhibit GLUT rather than the disruption of cytoskel-
eton proteins.

Classically, there are two types of Na^{+}/glucose
cotransporters (SGLT1 and SGLT2) [22], though an-
other, SGLT3, that is not a Na^{+}/glucose cotransporter, but
works as a glucose sensor, exists in the membrane of cho-
linergic neurons and skeletal muscles [49]. The expres-
sion of SGLT1 (the high-affinity transporter) has been
evertheless limited on the apical membranes of small-
intestinal absorptive cells and renal proximal straight
tubules. The apical membrane of renal convoluted prox-
imal tubules predominantly expressed SGLT2 (the low-
affinity transporter). There are no selective inhibitors
of SGLT, and phlorizin-related compounds are the only
available drugs for inhibiting Na^{+}/glucose cotransport via
SGLT. Further investigation will be needed in the future
to study SGLT classification by using a selective SGLT
inhibitor.

**Involvement of K_{ATP} and Ca^{2+} uniporter in the
regulation of pump lymphatic activity**

Ferrus et al. [50] reported that the synchronized
release of Ca^{2+} from the intracellular Ca^{2+} store in the
sarcoplasmic reticulum of lymphatic smooth muscles is
an important effector for producing spontaneous tran-
sient depolarization of the cells. Although Ca^{2+} gener-
ated from intracellular Ca^{2+} stores in smooth muscle
cells significantly affects this activity [51], there is some
indication that Ca^{2+} from the mitochondria of lymphatic
smooth muscles could be involved. The present study
demonstrated that 5-HD (a selective mitoK_{ATP} blocker)
significantly reduced the frequency of pump activity, and
the responses were completely reversed in the presence
of ruthenium red (an inhibitor of mitochondrial Ca^{2+} up-
take). Moreover, a selective opener of mitoK_{ATP}, diazox-
ide, significantly increased the frequency. In the present
study we supposed the mechanism to be that closure of
mitoK_{ATP} inhibits K^{+} influx, and then mitochondrial Ca^{2+}
uptake is increased via ruthenium-red sensitive Ca^{2+} uni-
porter to maintain membrane potentials (Fig. 9, box A).
Thus diazoxide studies elucidated the contrary response
that the mitoK_{ATP} opening caused an inhibition of Ca^{2+}
uptake (Fig. 9, box B). These ion dynamics between cy-
tosol and mitochondria in lymphatic smooth muscle cells
seem to produce a decrease/increase in intracellular Ca^{2+}
concentration ([Ca^{2+}]) and finally affect the frequency of
pump activity (Fig. 9, boxes A and B). In cardiomyocytes,
diazoxide caused the depolarization of mitochondria and
decreased mitochondrial Ca^{2+} content; the diazoxide-
mediated effects were blocked by 5-HD [17, 52]. They
therefore concluded that opening mitoK_{ATP} prevents Ca^{2+}
overload in cardiac mitochondria.

We previously reported that a K_{ATP} opener, pinacidil,
inhibited the pump activity of isolated mesenteric lymph
vessels, and glibenclamide (a nonselective K_{ATP} blocker)
completely reversed the pinacidil-induced responses
[10]. Thus activation/deactivation of K_{ATP}, located on the
plasma membrane significantly contributed to the regu-
lation of lymphatic activity [8–13]. In the present study,
the vessels were pressurized for the production of sponta-
neous activity of smooth muscles in the thoracic ducts, as
well as previous studies [9–12]. These methods enabled
us to examine the mechanical activity of isolated lymph vessels with no agonistic stimuli. We used $3 \times 10^{-3}$ M 5-HD in the present study. The concentration was lower than that of the other investigators, who used $3-5 \times 10^{-4}$ M 5-HD, considered effective in blocking mitoK$_{ATP}$ [17, 52]. Moreover, $10^{-3}$ M diazoxide used in the present study was around the threshold concentration that caused mitoK$_{ATP}$ to open [17, 52]. Our preliminary studies indicated that higher concentrations of 5-HD or diazoxide caused an irreversible effect on pump activity of the thoracic ducts or disrupted stable constriction/dilation of the vessels; therefore the effective concentration of mitoK$_{ATP}$ blocker/opener was accepted for use in the present study. On the other hand, the concentration of ruthenium red in the present study was similar to that used in other reports [34]. The pharmacological inhibition of mitoK$_{ATP}$ depends on how the channels are activated [18]. The drug selectivity of the target may also be altered under ischemic conditions, either as a result of altered high-energy phosphate content or changes in pH [21]. Inoue et al. [53] also reported that the single K$^+$ selective channel was inhibited by ATP applied to the channel’s mitochondrial matrix face (Fig. 9, box A). The studies that made use of FCCP (a protonophore of mitochondrial proton pump action) further indicate that in the present study, mitochondrial functions including the production of ATP are definitely involved (Fig. 9, box C). Because FCCP is a classical protonophore, preventing the maintenance of mitochondrial membrane potential at negative level and the concentration in the present study is within the effective range from $10^{-8}$ to $10^{-6}$ M FCCP [54, 55]. Therefore experimental conditions such as oxygen tension and pH or the cellular metabolic state may affect the activity of mitoK$_{ATP}$ in lymphatic smooth muscles as well as in other cells.

Glucose and mitoK$_{ATP}$-Ca$^{2+}$ uniporter in lymphatic pump activity

Figure 9 illustrates a schema of the glucose-ATP production pathway and mitochondria ion dynamics suggested by the present study. GLUT/SGLT located on the plasma membrane of lymphatic smooth muscles permit glucose to enter the cells. Glucose-6-phosphatase produces intracellular glucose from glycogen in a case such as the absence of extracellular glucose. Changes in the extracellular concentration of glucose, treatment of chlorenic acid, cytochalasin B, or phlorizin affected both the frequency of pump activity and diameter of the thoracic duct, whereas 5-HD/diazoxide only altered the frequency. Thus ATP produced by mitochondria may play a significant role in the regulation of both the frequency of pump activity and the contractility of lymphatic smooth muscles; the mitoK$_{ATP}$-Ca$^{2+}$ uniporter pathway partially modulates only the frequency. Under the present experimental conditions, the mitoK$_{ATP}$ gate in lymphatic smooth muscle cells may be opened and closed alternatively and partially because 5-HD or diazoxide itself affects the activity. MitoK$_{ATP}$-dependent regulation may be associated with ATP-mediated negative feedback mechanisms; the mitochondria-derived ATP not only facilitates the lymphatic pump, but also eliminates the functions. Lastly, we suggest that the lymphatic system is characterized by the effective transport and utility of glucose and that functional interaction between the mitoK$_{ATP}$ and Ca$^{2+}$ uniporter is involved in the regulatory mechanisms because of the low oxygen environment.

This study was supported by Grants-in-Aid for Scientific Research (17500303, 18500356) from the Japanese Ministry of Education, Science, Sports and Culture.

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