Stretch-induced calcium sensitization of rat lymphatic smooth muscle

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Shirasawa, Yuichi, and Joseph N. Benoit. Stretch-induced calcium sensitization of rat lymphatic smooth muscle. *Am J Physiol Heart Circ Physiol* 285: H2573–H2577, 2003.—The relationships between smooth muscle calcium and isometric tension generation to spontaneous lymphatic pump activity and its modulation by stretch equivalent from and isometric tension generation to spontaneous lymphatic pump activity were investigated. Excised preparations of pump activity and its modulation by stretch equivalent from were normalized and stabilized at a preload equal to 3 cmH2O, the peak generation in tension occurred 0.70 ± 0.11 s after that of calcium. Incremental stretch enhanced the frequency of the phasic activity and amplitude of isometric force generation but not the basal calcium level or the amplitude of the calcium transient. These findings suggest that stretch enhances lymphatic pump activity by increasing the pacemaker activity and the calcium sensitivity of the contractile apparatus.

lymphatics; lymphangion; thoracic duct

**The Lymphatic System** serves an important role in transporting interstitial fluid, proteins, macromolecules, and extravasated blood cells back to the systemic circulation. It is well established that lymph propulsion is dependent on interstitial fluid pressure and external compressive forces as well as active phasic contractions of the unidirectionally valved segments called “lymphangions,” which exhibit periodic contractile activity that centripetally propels lymph (2, 4, 11, 26). In the collection and transportation of lymphatics that are not embedded in parenchymal tissue (e.g., cisterna chyli and the thoracic duct), spontaneous lymphatic contraction is the predominant active mechanism of lymph propulsion.

The active contractile properties of lymphatic vessels have been widely studied. Contractility of lymphatic smooth muscle can be modulated by extrinsic (neural and humoral) and intrinsic (myogenic) factors. Pressure-induced modulation of lymphatic pump activity has been reported in bovine mesenteric (3–5, 8), and rat iliac lymphatic vessels (17). Modulation of calcium release from the intracellular stores has been suggested to be involved in the pressure-induced activation of lymphatic contraction (2). While the electromechanical events associated with lymphatic contraction remain unclear, the pacemaker activity has been attributed to an outward chloride current that is activated by calcium released from the sarcoplasmic reticulum (31, 32, 33). Like other types of smooth muscle, the action potential is mediated by calcium influx across the sarcolemma (15, 22). External calcium is also important for force generation (1, 15) and may exert modulatory roles in contraction frequency (15).

Despite the large amount of literature on lymphatic contractile function, few studies have directly assessed the relationship between calcium mobilization and lymphatic pump activity. Furthermore, there have been no reports on the effects of stretch on calcium and force transients in spontaneously active lymphatic pumps. Recently, we (30) successfully used a small wire myograph for simultaneous measurement of calcium and tension transients in small arteries. The purpose of the present study was to adapt the use of a small wire myograph to lymphatic vessels and to evaluate cytosolic calcium/force relationships in lymphatic smooth muscle at rest and in response to incremental stretch. A preliminary description of the method has been included in an abstract presented in the 2002 Experimental Biology meeting (28).

**METHODS**

A total of seven male Sprague-Dawley rats (Charles River Laboratories) weighing 439 ± 7 g was used in the study. Rats were anesthetized with isoflurane (Isoflo, Abott Laboratories) and euthanized by thoracotomy and transection of the cardiac ventricles. Procedures involving animals were reviewed and approved by the University of North Dakota Animal Care and Use Committee. The thoracic duct was excised and placed in cold (4°C) physiological saline solution (PSS; pH 7.4) containing (in mM) 119.0 NaCl, 4.7 KCl, 2.5 CaCl2, 1.2 MgSO4, 25.0 NaHCO3, 1.2 KH2PO4, 0.027 EDTA, and 5.5 glucose. With the aid of a stereomicroscope (Nikon), a segment of the cranial-most portion of the thoracic duct ~2 mm in length was dissected free of connective tissue. The vessel...
would equal 3 cmH2O. This normalization process accounted for the use of this relationship, the distance between the wires resembled a passive length-tension relationship. With the aid of curve was approximated by regression analysis. This plot was plotted as a function of internal circumference. An exponential curve from distance between the wires and their diameters and internal circumference (in mm) produced an increase in tension of 0.015 mN/mm. The vessel was held for 100 s at each length of stretch to allow force to become constant. This process was repeated until the calculated pressure reached ~6 cmH2O. The load on the vessel was equated to effective distending pressure \( P_{\text{effective}} \) in cmH2O according to a Laplace relation:

\[
P_{\text{effective}} = 2\pi \times \text{tension} \times \text{internal circumference}
\]

where tension = force (in mN)/(2 × segment length (in mm)) and internal circumference (in mm) = circumference calculated from distance between the wires and their diameters (40 \( \mu \)m) with the assumption of elliptical geometry.

The basal tension read from the myograph interface was plotted as a function of internal circumference. An exponential curve was approximated by regression analysis. This plot resembled a passive length-tension relationship. With the use of this relationship, the distance between the wires was set at the length where the effective distending pressure would equal 3 cmH2O. This normalization process accounted for interpreparation differences in segment length and diameter, thereby allowing us to study each lymphatic vessel under identical conditions at distending pressures similar to that occurring in the intact animal.

The ratio of fluorescence measured at excitation wavelengths of 405 and 485 was used as an index of cytosolic calcium. Calcium and force signals were continuously monitored and simultaneously recorded by a personal computer at 30 data points/s for later analysis. The frequency of the phasic activity at each step was evaluated by averaging interval times of calcium transients. Any chemical whose source was not mentioned was purchased from Sigma (St. Louis, MO).

**Data analysis.** The 405-to-485-nm indo-1 fluorescence ratio was used as an index of cytosolic calcium. Tension is reported as milliNewtons per millimeter. Data are means ± SE. Relationships were analyzed using standard regression analysis.

**RESULTS**

Figure 1 depicts representative tracings showing the relationship between calcium and tension in a thoracic duct segment. The vessel preparations used in this study all exhibited spontaneous rhythmic contractile activity. A calcium transient was associated with every measurable phasic contraction. The increase in calcium always preceded the increase in force. At a preload equivalent to 3 cmH2O, peak tension occurred 0.70 ± 0.11 s after peak calcium. Other profiles of the normalized vessels are summarized in Table 1.

Figure 2 shows representative tracings of tension and calcium in a thoracic duct subjected to incremental stretch. Each stretch produced an increase in isometric tension and was followed by a stable level of basal tone and spontaneous phasic contractions. The increase in stretch was not accompanied by an increase in calcium.

Figure 3 shows the relationship between average steady-state tension and internal circumference. Steady-state tension represents the average tension over the last 40–60 s of each stretch period. The relationship between tension and internal circumference could be approximated mathematically such that tension = 0.0016e\(^{0.0039\times \text{internal circumference}} \) \( (p^2 = 0.99) \).

Figure 4 shows the effects of increasing preload on contraction frequency, amplitude of the tension transient, basal calcium, and amplitude of the calcium transient. Both contraction frequency and the amplitude of tension generation increased linearly as a function of stretch. Neither basal calcium nor the amplitude of the calcium transient increased with stretch.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of vessels</td>
<td>7</td>
</tr>
<tr>
<td>Normalized diameter, ( \mu )m</td>
<td>259.7 ± 53.0</td>
</tr>
<tr>
<td>Maximum tension, mN/mm</td>
<td>0.128 ± 0.011</td>
</tr>
<tr>
<td>Basal tension, mN/mm</td>
<td>0.062 ± 0.011</td>
</tr>
<tr>
<td>Tension amplitude, mN/mm</td>
<td>0.066 ± 0.067</td>
</tr>
<tr>
<td>Contraction frequency, contractions/min</td>
<td>4.54 ± 0.68</td>
</tr>
</tbody>
</table>

Values are means ± SE.
DISCUSSION

The application of the small wire myograph for simultaneous monitoring of calcium and tension in spontaneous contractile lymphatics represents a major advancement in approaches to studying lymphatic smooth muscle. Although the isometric condition does not allow for evaluation of diameter changes, it does allow for precise measurements of force as well as calcium without the need to correct for cell volume changes associated with contraction. To our knowledge, this is the first report of its kind.

In the present study, each vessel segment was stretched to $6\text{ cmH}_2\text{O}$ of transmural pressure to obtain the passive mechanical properties of the lymphatic vessel. The range of distending pressures was based on prior measurements of right atrial pressure in normal Sprague-Dawley rats at rest ($3.6 \pm 0.5 \text{ cmH}_2\text{O}$) and during periods of severe edemagenic stress ($5.4 \pm 0.8 \text{ cmH}_2\text{O}$) (3). Thus we assume that the extent of stretch was within the range of pressures that occur in intact animals. The length-tension curve obtained in the present study could be fit to an exponential relationship (Fig. 3), as has been reported in other vessels (25). From this relationship, a transmural pressure point of $3 \text{ cmH}_2\text{O}$ was calculated for normalization in each vessel, which was previously reported as optimal for the pump activity of the rat thoracic duct (9). The contraction frequency after normalization ($4.60 \pm 0.95 \text{ contractions/min}$) agreed exactly with the value reported in isolated pressurized vessels under conditions of no flow ($4.6 \pm 0.6 \text{ contractions/min}$) (8). In addition, we could directly observe the temporal relationship between the spontaneous tension development of lymphatics and the accompanying calcium transient in a physiologically normalized condition. Calcium influx has been postulated to mediate the action potential associated with spontaneous lymphatic contraction (15). To this end, our data suggest that an action potential accompanies each phasic contraction.

Stretch increased both the frequency and amplitude of active spontaneous contraction (Fig. 4). This observation agrees with a previous report (7) of stretch-induced positive chronotropic and inotropic response of intact rat mesenteric lymphatics under edemagenic stress. Our findings in an isometric preparation, however, are different from those of Gashev and Zawieja (9), who used an isobaric preparation that showed maximal pacemaker activity at $3 \text{ cmH}_2\text{O}$ in the rat thoracic duct. These are interesting differences that do not appear to be linked to basal pacemaker activity, which were identical between the two studies. In the earlier study by Benoit et al. (5), it was postulated that both length-dependent and length-independent activation of lymphatic smooth muscle could contribute to the increased force and frequency of contraction of the lymphatic pump. The present study extends these findings by showing that the increased force of contraction occurs in the absence of an increase in cytosolic calcium. Stretch-induced active contractile responses have been widely reported in vascular smooth muscle. The response of lymphatic smooth muscle to stretch appears to differ from other types of vascular smooth muscle in which stretch-induced contraction has been chiefly attributed to elevation of intracellular calcium (10, 27). The major mechanism for vascular smooth muscle calcium elevation during stretch is presumed to involve release from intracellular stores in (6, 18,

Fig. 2. Representative recordings of calcium-dependent fluorescence and tension of a thoracic duct during passive stretch equivalent from 0 to $6 \text{ cmH}_2\text{O}$. Each arrow depicts where the vessel was stretched.

Fig. 3. Length-tension relationship of baseline tone in a rat thoracic duct segment. Each point depicts a mean value after each stretch with standard errors ($\bullet$, $n = 7$; $\square$, $n = 5$; $\triangle$, $n = 4$). Data series in each experiment were fit to an exponential curve ($r = 0.92 \pm 0.01$). T, steady-state tension; IC, internal circumference.
20) and/or inflow through voltage-dependent calcium channels on activation of cation channels (21, 34). In the present study, however, the amplitude and baseline of the lymphatic smooth muscle calcium were not altered by stretch. Thus we assume that the stretch-induced positive inotropic effect was calcium independent and that it was caused by increased sensitivity of the contractile elements to calcium. While little is known about the effect of stretch on the relationships between calcium and spontaneous contraction of lymphatic smooth muscle, several mechanisms for calcium sensitization in the myogenic response have been suggested in other types of smooth muscle. Those include myofilament overlap, steric change of associated proteins (7), and signaling pathways involving protein kinase C (12, 13, 29) and Rho kinase (14). It is interesting to note that both protein kinase C and Rho-A pathways are believed to alter calcium sensitivity. These examination of precise pathways in the modulation of lymphatic smooth muscle calcium sensitivity will require further study.

Previous studies (2, 5) by our laboratory have examined the effects of edema-promoting stimuli on lymphatic pumping in intact animals. Observed increases in frequency (chronotropic events) and ejection fraction (inotropic events) of intact lymphatics were directly correlated with the increased intralymphatic pressure resulting from the edema-promoting stimulus. The results of the present study agree with these earlier observations and indicate that moment-to-moment regulation of lymphatic pumping is highly dependent on the effective transmural pressure. Furthermore, we suggest that intrinsic regulation of lymphatic pumping involves a change in calcium sensitivity. These intrinsic events contribute to the ability of the lymphatic system to actively drain the interstitium in situations favoring enhanced lymph formation.

In summary, we examined calcium-tension relationships in the cranial part of the thoracic duct of the rat.
because this segment of the lymphatics is known to play an important role in the central propulsion of lymph (24). Our data suggest that stretch increases both the pacemaker activity and calcium sensitivity of the contractile apparatus. Future studies using the wire myograph will provide important new information regarding the adaptation of the lymphatic smooth muscle to chronic edemagenic stress.

**DISCLOSURES**

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**REFERENCES**