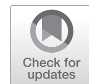

15 Lymphatic Vessel Pumping

Pierre-Yves von der Weid

Abstract

The lymphatic system extends its network of vessels throughout most of the body. Lymphatic vessels carry a fluid rich in proteins, immune cells, and long-chain fatty acids known as lymph. It results from an excess of interstitial tissue fluid collected from the periphery and transported centrally against hydrostatic pressure and protein concentration gradients. Thus, this one-way transport system is a key component in the maintenance of normal interstitial tissue fluid volume, protein concentration and fat metabolism, as well as the mounting of adequate immune responses as lymph passes through lymph nodes. In most cases, lymph is actively propelled via rhythmical phasic contractions through a succession of valve-bordered chambers constituting the lymphatic vessels. This contraction/relaxation cycle, or lymphatic pumping, is initiated in the smooth muscle cells present in the vessel wall by a pacemaker mechanism generating voltage-gated Ca^{2+} channel-induced action potentials. The action potentials provide the depolarization and Ca^{2+} influx essential for the engagement of the contractile machinery leading to the phasic constrictions of the lymphatic chambers and forward movement of lymph. The spontaneous lymphatic constrictions can be observed in isolated vessels in the absence of any external stimulation, while they are critically regulated by physical means, such as lymph-induced transmural pressure and flow rate, as well as diffusible molecules released from the lymphatic endothelium, perivascular nerve varicosities, blood and surrounding tissues/cells. In this chapter, we describe the latest findings which are improving our understanding of the mechanisms underlying spontaneous lymphatic pumping and discuss current theories about their physiological initiation.

Keywords

Lymphatic system · Lymphatic vessel · Lymphatic pumping · Lymphatic muscle cell · Lymphatic pacemaker · Spontaneous transient depolarization · Ca^{2+}-activated Cl^- channel · Intracellular Ca^{2+} store

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15.1 Introduction

The network of lymphatic vessels, or lymphatics, is widely distributed throughout the body and connects interstitial tissue space to lymphoid

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organs and to the blood circulation. It is through these vessels that the pool of fluid and proteins, which physiologically accumulates in the interstitium, is drained away in order to maintain tissue fluid balance. Once it enters the lymphatic vessels, the interstitial fluid is known as *lymph.* Unlike the cardiovascular system, which is a close circuit relying on a central pump to move blood, lymphatic vessels form a one-way system that collects lymph from the periphery and propels it, most of the time against hydrostatic pressure and protein concentration gradients [1], via the pumping action of successive individual lymphatic vessel chambers back into the venous circulation. In the process, lymph passes through several lymph nodes where particulate matter, antigens and immune cells interact with lymphocytes and participate in the immune surveillance.

This chapter first reviews the anatomy and functions of the lymphatic vessels, and then discusses our current knowledge of the specific aspects of lymphatic pumping and the mechanisms regulating the initiation of this spontaneous electrical and contractile activity.

15.2 Structural and Functional Organization of the Lymphatic Vessels

15.2.1 Initial Lymphatics

Interstitial fluid, along with various cellular components and proteins, enters the vessels through blind-ended structures known as *initial lymphatics*. The initial lymphatic vessels, also called *terminal* or *peripheral lymphatics*, or *lymphatic capillaries*, have walls devoid of muscle cells or pericytes. They are composed of a single layer of flattened, non-fenestrated endothelial cells laid on an incomplete basement membrane [2, 3], which overlap in an oak leaf-shaped manner.

Neighbouring endothelial cells are connected by button-like junctions made of vascular endothelial (VE)-cadherin and tight-junction proteins such as occluding, claudin-5, zonula occludens (ZO)-1, endothelial cell-selective adhesion molecule (ESAM) and junctional adhesion molecule-A (JAM-A). These junctions tightly join adjacent cells only at discrete locations [4]. The non-connected flaps of the endothelial cells form a primitive primary valve system, which when open creates 2–3 μm diameter pores allowing interstitial fluid to enter into the vessel lumen, and preventing leakage of lymph in the opposite direction [5–8]. Performance of these small valves is supported by *anchoring filaments*, collagenous fibrils linking the surface of the endothelial flaps to elastin fibers in the surrounding interstitial matrix [9]. These filaments prevent the thin-walled vessels from collapsing under high interstitial pressure, keeping them expanded to favor the entry of fluid, particulate matter, immune cells, proteins, chylomicrons [10, 11], as well as infectious pathogens such as bacteria and virus particles.

15.2.2 Collecting Lymphatics

Initial lymphatics empty their contents into collecting lymphatics, which connect with lymph nodes along their path and coalesce to form larger and larger vessels. Ultimately, lymph is returned to the venous circulation via the thoracic and the right lymphatic ducts which are connected to the left and right subclavian veins at the root of the neck.

The collecting vessels contain an intimal monolayer of endothelial cells, which unlike the initial vessels are tightly connected by zipper-like junctions that contain VE-cadherin, occluding, claudin-5, ZO-1, ESAM, and more evenly distributed JAM-A when compared with the initials [4]. The endothelial cells are surrounded by a basement membrane. Collecting lymphatics are distinguished from initials by the presence of a media comprised of one to three layers of smooth muscle cells intermixed with collagen and elastic fibers and an adventitia containing fibroblasts, connective tissue elements, and axons that innervate the vessel [12]. Tissue and species differences exist and, in smaller vessels, the three layers may not be easily distinguishable. However, as vessels progress centrally, the

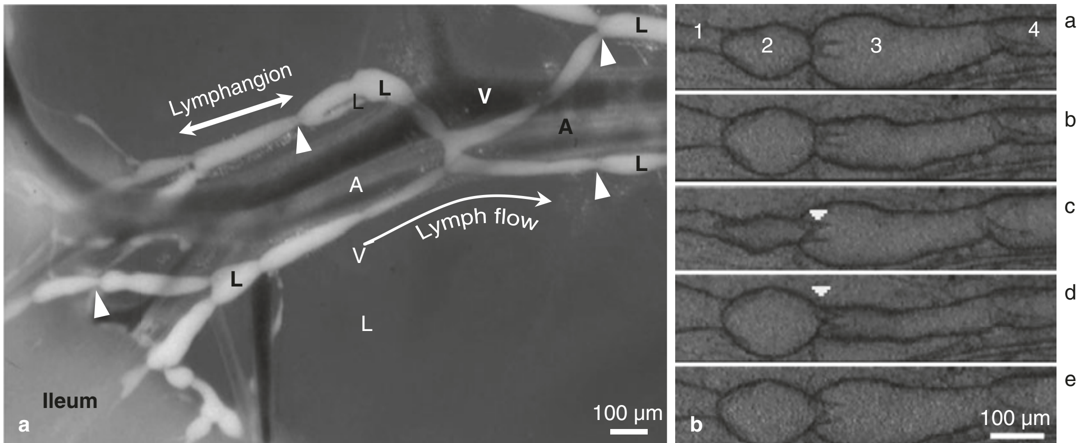

Fig. 15.1 Collecting lymphatic vessels in the guinea pig mesentery. (**a**) Mesentery containing ileum, artery (A), vein (V), and collecting lymphatic vessels (L), which are comprised of a succession of lymphangions, delineated by unidirectional valves (arrowheads). (**b**) Successive images of a lymphatic vessel under luminal perfusion. Four chambers (#1–4) are displayed, which underwent sequential contractions. A unidirectional valve (arrowhead) is visible between chambers #2 and 3

amount of smooth muscle increases, with fibers lying in a more circular orientation [13]. Another critical functional structure of the collecting lymphatics is the presence of unidirectional valves distributed along the length of the vessel. These valves consist of a matrix core sandwiched between endothelial cells and defined functional units termed lymphatic chambers or *lymphangions* [14] (see Fig. 15.1a).

Although extrinsic compression caused by skeletal muscle contraction, respiratory activity, and bodily movement also contributes, the main mechanism of lymphatic contraction is provided by the intrinsic contractile activity of the lymphatic smooth muscle which transiently and independently constricts each lymphangion. This phasic cycle of contractions and relaxations allows the lymph to be propelled into the next chamber through the downstream valve, while the generated fluid pressure closes the upstream valve limiting lymph backflow (see Fig. 15.1b). The ability of the lymphatic vessels to exhibit such rhythmic phasic constrictions is known as *lymphatic pumping* and is the mechanism whereby this vessel system performs its essential functions.

15.3 Roles and Functions of the Lymphatic Vasculature

15.3.1 Tissue Fluid Balance

In spite of daily variations in water and salt content, extracellular fluid volume is maintained remarkably constant. This consistency relies heavily on the control of transport of salts and fluid across the capillary wall, and the return of fluid to the plasma. About 90% of the fluid that leaks out of the blood circulation is reabsorbed into the venous system, and the remaining 10%, or about 5–6 L/day, enters the initial lymphatics and circulates as lymph through the human lymphatic vessels [15]. Lymphatics are also responsible for the daily clearance of about 60% of vascular proteins, as well as lipids and liposoluble vitamins incorporated into chylomicrons [10].

The largest determinants of fluid flux are the Starling forces across the capillary wall, namely *hydrostatic* and *colloid osmotic pressures*. Plasma and interstitial colloid osmotic pressure is determined by the concentration of solute molecules in these fluids. A higher plasma colloid osmotic pressure will favor osmotic absorption of fluid

into the capillary, because water will tend to move into an area of higher solute concentration. On the other hand, a high interstitial colloid osmotic pressure will favor an outward fluid flux in order to balance colloid concentrations across the wall. In most tissues, net colloid osmotic pressure drives fluid into the capillary, and the overall hydrostatic pressure forces fluid out into the tissue. The resulting net balance of forces tends to direct fluid out of capillaries and into the interstitium. Since lymphatic loading is directly proportional to interstitial fluid volume, the amount of lymph that is propelled in the vessels is also determined by the plasma volume filtrated into the interstitium; the greater this volume, the larger the amount of fluid that is transported via the lymphatics back into the bloodstream.

Protein transport out of capillaries must also be balanced with the same amount of proteins leaving the interstitium via the lymphatics. An inability to transport filtered proteins out of the interstitium results in edema. Proteins move across the capillary membrane by means of both diffusion and convection. There are several determinants of transvascular protein transfer, some which relate to characteristics of the capillary membrane itself, and others which relate to the relative concentrations of protein across the membrane. The flux of proteins across the capillary wall is regulated by a complex set of physiological mechanisms. This is important because fluid flux depends largely on osmotic pressure, and protein concentration can have a large impact on fluid volumes. As in fluid loading in lymphatics, the amount of protein loaded into the initial lymphatics is determined by interstitial protein concentrations. Lymph formation must match the net transcapillary flux of fluid and solutes in order to prevent excessive tissue swelling and edema [15].

15.3.2 Lymph Formation and Transport

Fluid, electrolytes, proteins, and cellular elements, such as immune cells, do not just passively drift into the lumen. The current theory proposes that the phasic expansion/relaxation phases generated by the lymphatic pump in the initial or downstream collecting lymphatics (see below) allow the transient development of hydrostatic pressure differences between the interstitium and the lumen of the initials that favor fluid entry [16–21]. The efficiency of lymph entry into the initial vessels and its central movement strongly rely on the competency of the initial and collecting valve systems [5, 22, 23]. Fluid movement through the tissue towards the lymphatic vessel lumen is vital to maintain vessel filling, and can only be preserved if the downstream lymphatic vessels continue to drain fluid away.

15.3.3 Lymphatic Vessels and Immunity

The lymphatic system is strongly implicated in the adaptive immune response. As part of this system, lymphatic vessels are responsible for transporting antigens to lymphoid tissues, where they are sequestered to bring about immune responses during disease and in response to infection. Antigens present in fluid entering the lymph node can effectively elicit an immune response upon activation of resident naïve T- and B-cells, which enter lymph nodes from tissues via afferent lymphatic vessels or from the cardiovascular network via high endothelial venules. Immune cells exit the lymphatic system and eventually return to the bloodstream to be transported throughout the body looking out for foreign antigens.

The lymphatic system provides an exclusive environment in which immune cells can respond to foreign antigens, as well as proliferate and circulate lymphocytes and return them to the bloodstream. Importantly, lymphatic vessels also serve as preferred routes for the spreading of metastatic cancer cells from the primary to different organs. Indeed, lymph nodes are regarded as a favorable environment for metastatic tumor development [24].

The lymphatic system also functions as a one-way communication system for molecular messages, such as cytokines and chemokines, which

can be transmitted to cellular constituents in lymph nodes. These messengers are also potential candidates for controlling lymph flow by modulating lymphatic pumping [25–27]. A major role for the lymphatic vessels has also been demonstrated in the transport of venom after snakebite, due to the usually large size of the toxic molecules [28, 29].

15.3.4 Digestive Functions

Following initial observations by Asselli of the key role lymphatics of the mesentery played in digestion and absorption (reviewed in [30]), it is now well recognized that intestinal lymphatics are crucial to the absorption of dietary lipids [31–34]. Fat absorption involves emulsification of bile salts, hydrolysis of long-chain triglyceride species by lipase, passive and/or transporter-mediated diffusion into enterocytes, and resynthesis and repackaging of large triacylglycerol-rich lipoproteins into chylomicrons. Chylomicrons are exocytosed by the enterocytes and enter the lymphatic system through lacteals, initial lymphatics in the villi of the small intestine, which empty into submucosal lymphatics and then into mesenteric collecting lymphatics. It is well accepted that intestinal lymph flow is enhanced following fat feeding [35, 36], but the precise mechanisms by which this occurs and the impact of lipid metabolism on lymphatic function still require further investigation.

15.4 Lymphatic Pumping and Spontaneous Contractions

Lymphatic pumping is the main driving force for lymph transport against adverse pressure gradients [37]. This active propulsive process is generated by the robust constriction/relaxation cycle of the lymphangions in the collectors, each lymphangion behaving like little ventricles in series. The centripetal and upstream movement of lymph is also facilitated by the system of one-way valves within the lymphatic network, which minimizes backflow [38].

15.4.1 Lymphatic Muscle Contraction

The lymphatic muscle cells invested in the walls of collectors generate and control the movement of lymph along the lymphatic network. As they bear similarities with vascular smooth muscle cells, lymphatic muscle cells are usually classified as smooth muscle cells. However, they display important differences in both their contractile function and contractile machinery that make them unique. Lymphatic contraction is mainly regulated by the rate of myosin light-chain phosphorylation/dephosphorylation, controlled by the balanced activity of myosin light-chain kinase (MLCK) and myosin light-chain phosphatase (MLCP), a mechanism well studied in blood vessels (reviewed in [39]). As intracellular Ca^{2+} ($[Ca^{2+}]_i$) increases, it binds to calmodulin, and the Ca^{2+}-calmodulin complex activates MLCK. MLCK phosphorylates the regulatory myosin light chain 20 (MLC_{20}) [40], causing activation of the myosin ATPase by actin and thus contraction. When $[Ca^{2+}]_i$ decreases, MLCK is deactivated leading to the dephosphorylation of MLC_{20} by MLCP, the myosin ATPase is deactivated, and the muscle relaxes. MLCK and MLCP activities and contractile status can also be regulated by mechanisms independent of $[Ca^{2+}]_i$ [40, 41]. Although these mechanisms explain well how tonic contraction is regulated, studies by Muthuchamy et al. [42] characterizing the contractile proteins expressed by lymphatic muscle cells shed some light on the mechanisms that could explain the ventricular-like pumping function of the lymphangions. They demonstrated that rat mesenteric and thoracic lymphatic muscle cells contain striated contractile elements and share biochemical and functional characteristics with cardiac muscle cells [42]. Specifically, lymphatic muscle cells express the SMB-MHC isoforms, as well as fetal cardiac/skeletal slow-twitch β-MHC, a faster non-smooth muscle MHC isoform, conferring them relatively

fast contractile properties. Furthermore, lymphatic tissues express messages and proteins for both vascular smooth muscle actin and sarcomeric actin (reviewed in [43]). Whether the striated muscle and smooth muscle isoforms are expressed in the same lymphatic muscle cells has not been determined yet.

15.5 Electrophysiological Properties of the Lymphatic Muscle

As mentioned earlier, rhythmical constrictions can be observed in isolated collectors maintained in physiological conditions in the absence of innervation or a functional endothelial layer [44, 45]. They are driven by action potentials generated by the cells constituting the muscular part of the vessel [46–48]. The regularity of these events suggests a pacemaking mechanism. While several molecular and electrical entities have been proposed to underlie the lymphatic pacemaker, the precise/complete characterization of its mechanisms is still under study. We discuss in the following sections studies that have forged our current understanding of the pacemaker activity driving lymphatic pumping.

15.5.1 Historical Perspective

Lymphatic muscle membrane potential and action potentials have been investigated with recording methods of increased complexity over the years. The first successful attempts were obtained by Mislin [49, 50] using extracellular electrodes. He was able to record a series of peaks of short duration followed by a slower "after-potential" that he referred to as electrolymphangiograms (ELG) and that he interpreted as action potentials preceding the lymphangion constriction. Measurements of lymphatic intracellular voltage changes were initially made with the sucrose gap technique. Although providing an approximate and dynamic description of intracellular voltage changes rather than absolute membrane potentials, this technique originally used by Orlov et al. [51] in bovine mesenteric lymphatics allowed the recording of the action potential time course as a single spike followed by a gradually declining plateau, further confirming the correlation between action potential and constrictions [46–48]. Sharp intracellular microelectrodes have subsequently been used, providing a more reliable measurement of the action potential and the first accurate measurement of the lymphatic muscle membrane potential.

15.5.2 Lymphatic Muscle Membrane Potential

Using intracellular microelectrodes, mean resting membrane potential values ranging from −66 to −48 mV were obtained from mesenteric lymphatic vessel preparations of the guinea pig [52–54], rat [55, 56], and bovine [57–59]. The small variation between values could be due to species or methodological differences or more plausibly relate to the consistently more active (and more depolarized) bovine and rat vessels compared to the generally more quiescent (and hyperpolarized) guinea pig lymphatics. The polarized values of these recorded membrane potentials probably reflect the fact that these measurements were obtained in mesenteric vessels in the absence of applied transmural pressure. More recent recordings have been successfully obtained from pressurized or stretched lymphatic vessels. Because of the higher action potential frequency induced by the vessel pressure/stretch, the measured membrane potential is more correctly defined as mean diastolic membrane potential, with reported values being more depolarized. Indeed, isolated rat mesenteric lymphatics mounted on a wire myograph displayed a mean diastolic membrane potential of −39 mV that did not significantly change with increase in vessel stretch [55]. Using the same wire myograph technique, Telinius et al. reported membrane potentials of ~−45 mV in human thoracic ducts [60]. A limitation of the wire myograph procedure is that, due to the small size of the vessels, the lym-

phatic endothelial layer may well be damaged by the wires inserted through the vessel lumen, compromising the release of nitric oxide (NO) and prostaglandins known to hyperpolarize the muscle. Furthermore, the horizontal and lateral stretch stimulus applied by the myograph might lead to different electrical response than the radial pressure normally experienced by the vessel. Thus, whether stretch truly depolarizes the lymphatic muscle cannot be unequivocally confirmed with this method. More recently, Davis et al. successfully obtained membrane potential recordings from pressurized mouse, rats, and human lymphatic vessels, where sharp, intracellular microelectrode impalements could be maintained when contractions were blunted with low millimolar concentration of the MLCK inhibitor wortmannin [61]. With this technique, the authors reported diastolic membrane potentials of −40 mV for rat mesenteric vessels, and −35 and −40 mV for mouse inguinal and mesenteric collectors pressurized to 3 cm H_2O [61, 62]. In addition to these values being more depolarized compared to unstretched vessels and not noticeably different from those obtained with a wire myograph, they were little altered (~3 mV) with increases in pressure (M.J. Davis, personal communication).

Based on pharmacological experiments performed on sheep and guinea pig mesenteric lymphatics, large-conductance Ca^{2+}-activated K^+ channels, inward-rectified K^+ channels, delayed rectifier K^+ channels, ATP-sensitive K^+ channels, and Ca^{2+}-activated Cl^- channels (Cl_{Ca}) have been suggested to be expressed and involved in the establishment of the lymphatic muscle membrane potential [63–67]. The functional contribution of all these classes of K^+ channels was also more recently demonstrated in human thoracic ducts and their expression confirmed at the mRNA level [68]. The difficulty to acutely isolate lymphatic muscle cells has limited the use of the patch clamp technique to characterize the currents involved in lymphatic muscle membrane potential, so that only a very few studies using this technique have been published. Thus far, a large-conductance Ca^{2+}-activated K^+ current (blocked by penitrem A) and several components of voltage-dependent K^+ currents are characterized by their partial blockade by TEA or 4-AP, thereby resembling "delayed rectifier" currents identified in acutely dissociated sheep mesenteric lymphatic muscle cells [64]. Furthermore, a nifedipine- and Bay K8644-sensitive L-type Ca^{2+} current and a 9-anthracene carboxylic acid (9-AC)-sensitive Cl_{Ca} current activated by Ca^{2+} release from intracellular stores were recorded from a significant number (~70%) of the same isolated sheep mesenteric lymphatic muscle cells [66].

Several electrogenic ion pumps and exchangers have also been suggested to be involved in the establishment of the lymphatic muscle resting membrane potential. Inhibition of the sodium-potassium-ATPase with ouabain causes a 6–10 mV depolarization in bovine and guinea pig mesenteric lymphatics [57] (von der Weid, unpublished data). Similarly, a hyperpolarization of ~6 mV is observed in rat mesenteric lymphatics during inhibition of the Na^+–K^+–$2Cl^-$ co-transporter with bumetanide [56], which correlates with a significant decrease in contraction frequency (Fig. 15.2). Together with the reported sensitivity of the resting membrane potential to ion substitution and ion channel inhibition [56, 69], these data demonstrated the strong lymphatic muscle membrane potential dependency to K^+ and Cl^- conductances.

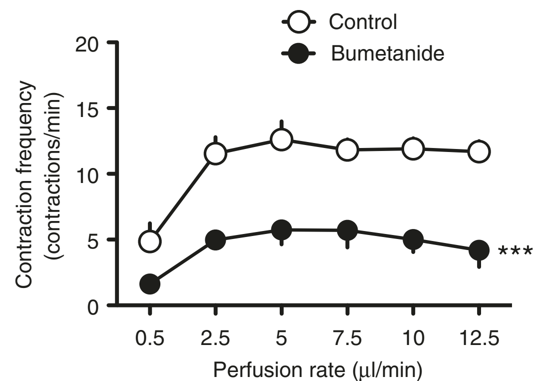

Fig. 15.2 Effect of the Na^+–K^+–$2Cl^-$ co-transporter (NKCC) blocker bumetanide (10 μM) on the perfusion-induced increase in contraction frequency of guinea pig mesenteric lymphatic vessels ($n = 7$; ***$p < 0.001$, two-way ANOVA with Bonferroni posttest)

15.5.3 Action Potential

Due to their frequent occurrence and their importance in lymphatic contractions, characterization of the electrophysiological mechanisms involved in the action potential and the initiation of this event has been the subject of much attention. In bovine mesenteric vessel, intracellular microelectrode recordings showed a diastolic slow depolarization and followed by a spike complex superimposed to a plateau phase [70]. Similar spike complexes were reported in sheep and human mesenteric collectors [71, 72]. While action potentials in guinea pig mesenteric lymphatics displayed the initial transient depolarization and plateau phases, a slow depolarization and superimposed burst of spikes were not evident in this species [73]. Spontaneous action potentials were more recently recorded from mouse main collectors [61]. In this study, the authors reported differences in action potential properties between lymphatic vessels in the leg and vessels from the visceral cavity. While inguinal/axillary action potential was characterized by a diastolic depolarization, spike, plateau, and repolarization phases without a noticeable after-hyperpolarization, mesenteric action potentials lacked spike and plateau phases. This difference, partially explained by L-type Ca^{2+} channel activity, is likely correlated with the notorious lack of contractile activity of mouse mesenteric collectors [61].

Like most smooth muscles, action potentials and constrictions in lymphatics depend heavily on extracellular Ca^{2+} ions and are inhibited by L-type Ca^{2+} channel blockers [47, 74, 75]. Electrophysiological evidence for L-type Ca^{2+} current was confirmed in freshly isolated sheep mesenteric smooth muscle cells with the whole-cell patch clamp method [66, 76]. Involvement of L-type Ca^{2+} channels in rat, mouse and human mesenteric vessel action potentials was assessed pharmacologically [60, 61, 77] and expression of the main L-type Ca^{2+} channel isoform, Cav1.2, confirmed by PCR and immunofluorescence [60, 61, 77], although expression of Cav1.1 and Cav1.3 was also revealed.

A fast voltage-activated Na^+ current, inhibited by TTX and which contributes to the rising phase of the action potential, was also identified in the sheep mesenteric smooth muscle cells [78]. TTX also significantly depressed spontaneous contractions of isolated sheep mesenteric lymphatic vessel rings suggesting the involvement of Na^+ channels in spontaneous constrictions [78]. However, TTX did not affect action potentials in bovine mesenteric lymphatics [47] or constrictions induced by luminal perfusion in guinea pig mesenteric lymphatics [52]. Telinius and colleagues, using pharmacological characterization and mRNA expression profiling, reported expression of multiple voltage-activated Na^+ channels (Nav1 family) in samples of human thoracic duct and mesenteric lymphatic muscle with Nav1.3, the most prevalent, facilitating the action potential generation [72].

15.5.4 Lymphatic Pacemaker

The strict voltage dependence of L-type Ca^{2+} channels and the regularity of the action potentials during lymphatic pumping suggest the involvement of an electrical event that transiently depolarizes the muscle membrane potential (pacemaker potential). Two main mechanisms have been proposed for the generation of pacemaker activity in lymphatic vessels.

Studies on large lymphatic vessels (bovine mesenteric lymphatics) have demonstrated that the pacemaker mechanisms underlying lymphatic constrictions relate to a slow depolarization leading to the generation of regularly occurring action potentials [48, 70]. Such activity bears time- and voltage-dependent similarities with that observed in the heart. Indeed, investigations in sheep mesenteric lymphatics have demonstrated the existence of a hyperpolarization-activated inward current with properties similar to hyperpolarization-activated cyclic nucleotide-gated (HCN or “funny” current I_f) current in the sinoatrial node [79]. This current could however be only recorded in a low number of cells. HCN blockers, cesium and ZD7288, partially inhibited the frequency of spontaneous constrictions of isolated lymphatic segments. A more profound effect of the selective HCN

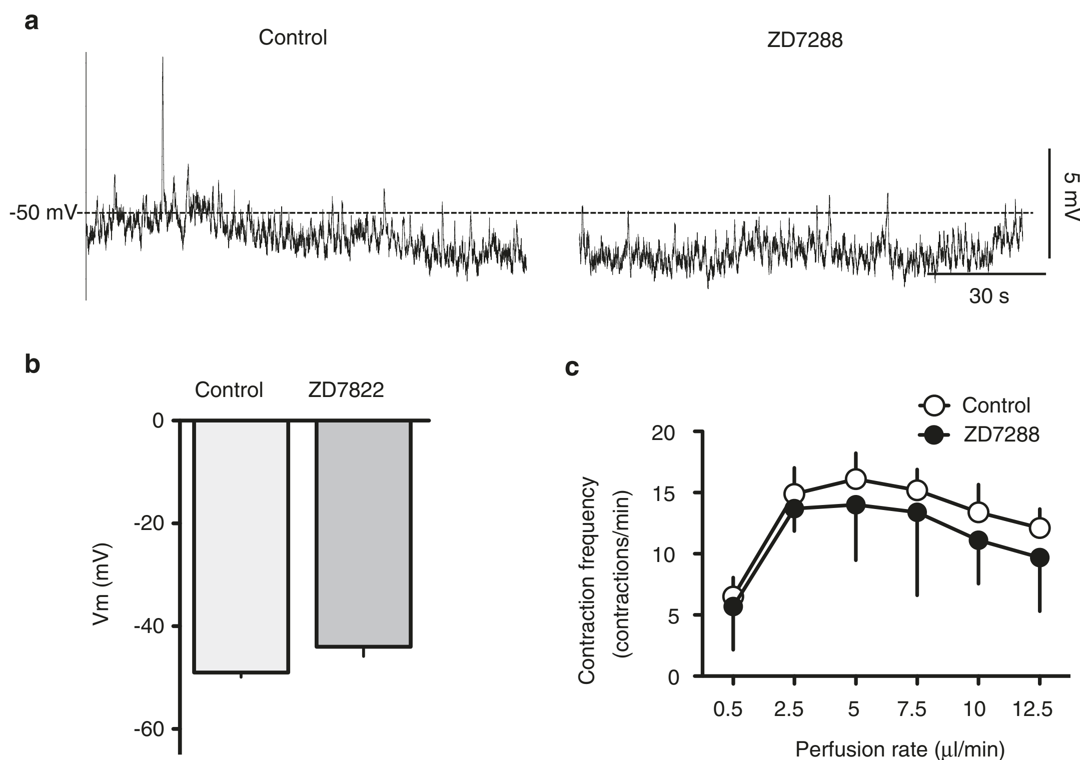

Fig. 15.3 Lack of effects of the HCN channel blocker ZD7288 on membrane potential and contractile activity of guinea pig mesenteric lymphatic vessels. Original intracellular microelectrode recording of membrane potential in control condition and 10 min after the start of a 1 µM ZD7288 superfusion (**a**: note small and large upward deflections denoting STDs), and summary graph data (**b**: n = 4). (**c**) Contractile responses of guinea pig mesenteric lymphatic vessels luminally perfused at increasing flow rates in control condition and in the presence of 1 µM ZD7288 (n = 2)

blocker ivabradine, as well as to a lesser extent cesium and ZD7288 (at rather high concentrations), to inhibit lymphatic pumping was more recently reported in rat diaphragmatic lymphatics, which express all four HCN channel isoforms [80]. However, ZD7288 exhibited no significant effects on the membrane potential of guinea pig mesenteric lymphatic muscle, nor did it alter the frequency of action potentials and constrictions in rat and guinea pig mesenteric vessels (Fig. 15.3 and von der Weid, unpublished data), or the activity of spontaneous transient depolarizations (STDs) [69], the events proposed to mediate pacemaking in these vessels (see below). Altogether, these findings suggest that while a HCN conductance may be important for lymphatic pacemaking in several vessels or species, it plays a lesser role in others, where additional conductances predominate.

An issue associated with pacemaker potentials that has hampered their characterization is the electrical characteristics of the muscle syncytium present within the lymphatic chamber. In large vessels, pacemaker activity is difficult to study because of uncertainties in the electrical distance of the pacemaker cells from the site at which recordings are made in the smooth muscle. Thus, the pacemaker potential that generates each action potential cannot readily be distinguished from the potential change, underlying the propagated action potential. By studying smaller, more segmented vessels in the guinea pig mesentery, van Helden circumvented this problem and proposed a second model for lymphatic pacemaking. In these small, presumably isopotential short vessel segments, lymphatic muscle membrane potentials recorded with a intracellular microelectrode consistently displayed small STDs that

were proposed to be the lymphatic pacemaker, because either individually or through summation they underlie the action potentials and muscle contractions [53]. This hypothesis is supported by a number of observations reported in this original paper and subsequent studies [69, 81–84]. First, the initial phase of the spontaneously generated action potentials has the same time course as the rising phase of STDs. Second, agonists, such as noradrenaline, histamine, endothelin-1, or thromboxane A_2 mimetic U46619, which all increase lymphatic pumping rate, enhance STD activity. Third, STDs occur independently of both the innervation and the endothelium, making them likely to be generated by the lymphatic muscle cells. STDs reflect the opening of Ca^{2+}-activated inward current carried by Cl^- ions upon the "packeted" release of Ca^{2+} from IP_3-sensitive stores within the muscle cells [69]. In support of these findings, Toland et al. identified a Cl_{Ca} current in isolated sheep mesenteric lymphatic smooth muscle cells, using the perforated patch technique [66]. The authors demonstrated that while in current clamp mode inhibition of this current with 9-AC reduced STDs and action potentials, the same treatment also caused abolition of pumping in a pressurized vessel. This hypothesis that Cl_{Ca} channels underlie STDs and participate in the lymphatic pacemaking is also in agreement with the growing body of evidence showing that Cl_{Ca} channels are involved in agonist-induced rat vascular smooth muscle contraction [85, 86].

Elusive for a long time, the identity of the gene underlying these Cl_{Ca} channels has now been determined as anoctamin-1 (*Ano1*), also known as TMEM16a. ANO1 channels have been recently shown to be expressed in human, mouse, and rat lymphatics [87] (S.D. Zawieja and M.J. Davis, personal communication; von der Weid, unpublished data) and Cl_{Ca} currents have been recorded from lymphatic muscle cells acutely dissociated from mouse inguinal-axillary collectors (S.D. Zawieja and M.J. Davis, personal communication). The important role of ANO1 channels in lymphatic pumping was further demonstrated by the strong reduction of lymphatic contraction frequency and a lack of response to increase in transmural pressure after selective deletion of the *Ano1* gene in mouse lymphatic muscle cells (S.D. Zawieja and M.J. Davis, personal communication). In these transgenic mice, the lymphatic muscle cells were hyperpolarized, lacked the characteristic diastolic depolarization, and had a blunted action potential plateau, a time course similar to that reported in sheep lymphatic muscle cells treated with 9-AC [66]. Despite the strong evidences that STDs underlie lymphatic pacemaking and that STD inhibition correlates strictly with abolition of action potentials, several observations challenge the STD hypothesis for lymphatic pacemaker. Although it is likely that other conductances such as HCN currents also contribute, it is troubling that while Cl_{Ca} channel blockers, niflumic acid or 9-AC, are very potent at inhibiting STDs [66, 69], they only slightly decrease the frequency of contractions [55] (see Fig. 15.4). The argument that the notoriously nonspecific actions of these Cl_{Ca} channel blockers may also target other channels, such as L-type Ca^{2+} channels, is refuted by the fact that if the contraction frequency is not affected, neither should the action poten-

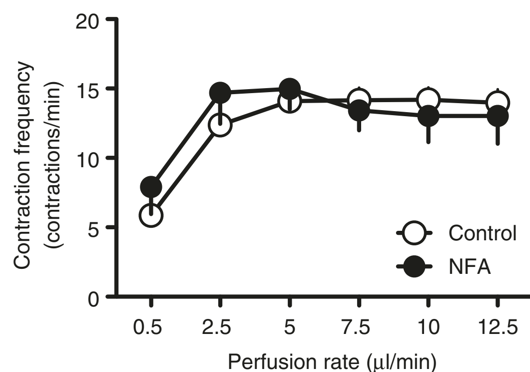

Fig. 15.4 Lack of effect of the Ca^{2+}-activated Cl^- channel blocker niflumic acid (NFA; 50 μM) on the perfusion-induced increase in contraction frequency of guinea pig mesenteric lymphatic vessels ($n = 7$)

tials. Moreover, although lymphatic pumping is markedly impaired in the pressurized ANO1$^{-/-}$ transgenic vessels, it is not totally abolished, thus aligning well with the finding that niflumic acid did not alter action potential frequency in wire myograph-mounted rat mesenteric lymphatics [55]. A careful examination of the possible reasons for the differences in the effects of the Cl_{Ca} channel blockers points to the different experimental conditions used for the different studies. In order to allow electrical recordings with sharp microelectrodes, inhibition of STDs was mostly assessed in quiescent, unstimulated vessel segments, displaying what we can consider to be true "spontaneous contractile activity." On the other hand, assessment of the effects of these blockers on lymphatic pumping was typically performed on isolated vessels under pressure/stretch conditions. It is legitimate to suggest that the strong mechanical stimulation activates another rhythmical mechanism, and to hypothesize that pressure-induced pacemaking requires activation of a set of ion channels different than those underlying spontaneous pacemaking.

It is well known that the degree of distension or stretch of the lymphatic vessel wall is an important factor in determining the intrinsic contractile ability of these vessels and hence the propulsion of lymph. Raising transmural pressure in doubly cannulated mesenteric lymphatic vessels causes an increase in both constriction frequency and amplitude and thus an increase in the amount of fluid propelled [88]. Benoit et al. [89] reported an increase in volume and constriction frequency of intact mesenteric lymphatics of the rat during edemagenic stress created by plasma dilution and concluded that a stretch-dependent mechanism was involved. Although STD frequency was shown to increase with stretch in rat mesenteric lymphatics mounted on a wire myograph [55] and with intraluminal flow [82, 90] other ion channels may underlie the response to stretch or pressure.

15.5.5 Roles of Intracellular Calcium in the Lymphatic Pacemaker

In addition to being the ion permeating through L-type channels to generate action potential and excitation-contraction (E–C) coupling [91], Ca^{2+} released from intracellular store is also involved with lymphatic pacemaking [65, 66, 82, 92–94].

Atchison et al. [93] first reported that intracellular Ca^{2+} store modulators, caffeine, ryanodine, and cyclopiazonic acid (CPA), all inhibited lymphatic pumping in actively-contracting isolated bovine mesenteric lymphatic vessels, implicating intracellular Ca^{2+} stores (including a ryanodine sensitive store) in the phasic contractile activity. When transmural pressure was increased the inhibition evoked by caffeine and CPA was greater than that produced by ryanodine. This observation led to their suggestion that stores other than the ryanodine-sensitive stores were also involved. This work is concordant with studies on guinea pig mesenteric vessels, which shows that STDs are not blocked in Ca^{2+}-free solution, but inhibited by either BAPTA-AM to chelate $[Ca^{2+}]_i$ and or CPA to inhibit Ca^{2+} reuptake by the sacro-endoplasmic Ca^{2+} ATPase (SERCA). In this preparation, STDs were untouched by treatment with ryanodine or tetracaine [69], pointing to the specific contribution of IP_3-sensitive stores in their generation [53, 92]. This conclusion is further confirmed by the observations that IP_3 receptor (IP_3R) blockers, 2-aminoethoxydiphenyl borate (2-APB) and xestospongin C, reduced STD amplitude. Thimerosal, known to sensitize IP_3R for IP_3, and Bt3(1,3,5)IP_3/AM, a membrane-permeant analog of IP_3, increase STD amplitudes, further confirming the specific involvement of IP_3-sensitive Ca^{2+} stores [69]. Importantly, 2-APB has no significant effect on contraction and action potential frequency of myograph-mounted rat mesenteric lymphatics (Fig. 15.5), again suggesting that different/additional molecular elements might be involved in pacemaking when vessels are activated by wall stretch.

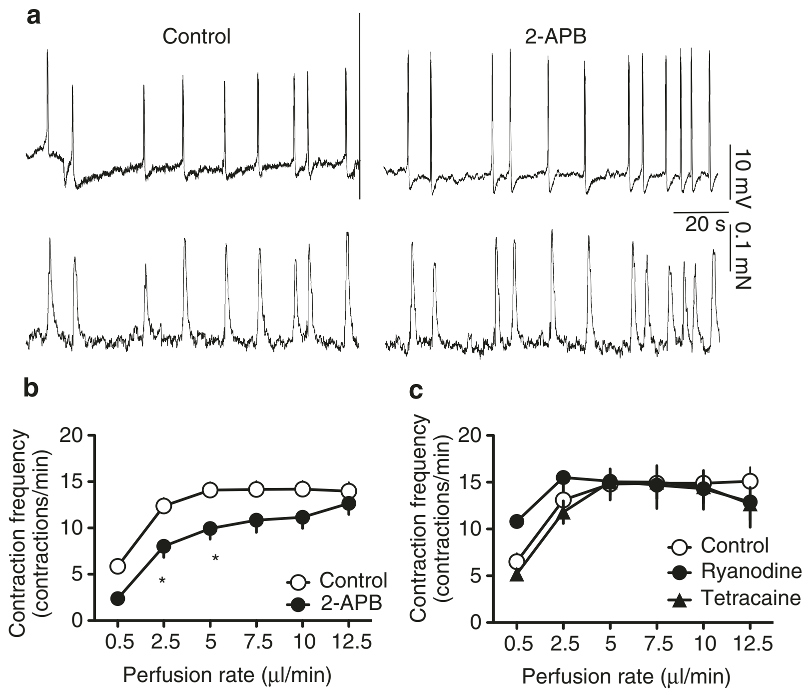

Fig. 15.5 Minimal involvement of intracellular Ca^{2+} stores in pacemaking of stretched lymphatics. (**a**) Original simultaneous recordings of membrane potential (top traces) and force (bottom traces) under control conditions (left) and 10 min after addition of 50 µM 2-APB to the solution bathing rat mesenteric lymphatic vessels mounted on a wire myograph under a preload of 0.3 mN. Traces are representative of five experiments. (**b**, **c**) Contractile responses of guinea pig mesenteric lymphatic vessels luminally perfused at increasing flow rates in control condition and in the presence of 50 µM 2-APB ($n = 13$; **b**) and 30 µM ryanodine ($n = 5$) or 50 µM tetracaine ($n = 5$; **c**)

15.5.6 Spontaneous and Stretch-Induced Pacemaker

A model for lymphatic pacemaking proposed by Imtiaz and colleagues can be used as a starting point to reconcile differences in pacemaker characteristics observed in unstretched and stretched vessels. In their model, the authors propose that the interactions of two coupled oscillators account for the rhythmic lymphatic pacemaker [84, 94]. The first oscillator is composed of the interaction of IP_3 with IP_3R and the subsequent sensitization of IP_3R by Ca^{2+} that is released internally. The second oscillator is represented by the electrical membrane potential changes leading to action potential firing in one cell that spreads and triggers depolarization and action potentials in adjacent cells coupled through gap junctions. Nonselective gap junction inhibitors have established a critical role for gap junctions in maintaining coordinated intra- and inter-lymphangion contractions in pressurized lymphatic vessel studies [95–97], but the identity of the various connexins mediating coupling between lymphatic smooth muscle cells is unknown at this time.

The limited ability of 2-APB, as well as ryanodine, to disrupt the contractile rhythmicity of lymphatic vessels under stretch [93] (see Fig. 15.5) suggests that the contribution of Ca^{2+} released from intracellular Ca^{2+} stores to pacemaking lessens as pressure/stretch of the vessel wall increases. However, as contractility strongly increases with pressure, the second, membrane-based, oscillator becomes more important. The ability of the cell membrane to sense and respond to increases in intraluminal pressure is critical and certainly involves the activation of a mecha-

nosensor. While the molecular identity of this structure is still unknown, it presumably involves the opening of ion channels to provide the depolarization and/or Ca^{2+} entry necessary for the direct or indirect initiation of action potentials and contraction. We thus hypothesize that under stretch, activation of the mechanosensor and the ensuing depolarization and/or Ca^{2+} entry take over the mechanism of initiation of spontaneous action potential/contraction, exemplified by STDs, to increase lymphatic pumping.

A study by Lee et al. investigating the role of voltage-dependent Ca^{2+} channels in stretch-induced lymphatic pumping showed a critical contribution of T-type voltage-dependent Ca^{2+} channels in pacing contractions generated by stretch [77]. Specifically, the authors revealed a significant inhibition of the increase in frequency of action potentials and contractions generated by stretch or transmural pressure in rat mesenteric lymphatics when vessels were treated with T-type voltage-dependent Ca^{2+} channel blockers, Ni^{2+} or mibefradil. These findings strongly contrasted with the effects of L-type voltage-dependent Ca^{2+} channel blockers, nifedipine and diltiazem, which significantly decreased only the force of contractions [77]. In addition to demonstrating the important role of T-type Ca^{2+} channels in lymphatic pacemaking, the study also revealed that Ni^{2+} and mibefradil differentially modulated lymphatic muscle membrane potential in wire myograph-mounted lymphatic vessels and unstretched vessels. Administration of the T-type Ca^{2+} channel blockers on unstretched vessels where lymphatic muscle membrane potentials ranged from −55 to −65 mV revealed a significant hyperpolarization, suggesting a contribution of these T-type Ca^{2+} channels to the resting membrane potential. Indeed, these channels are classified as low-voltage activation channels, which open at polarized membrane potentials [98]. On the other hand, when applied to lymphatic segments mounted on the wire myograph where muscle cells are more depolarized (−35 to −45 mV) [55], these blockers caused no significant changes in membrane potential, while inducing a decrease in contraction frequency. The involvement of T-type Ca^{2+} channels in the regulation of membrane potential in unstretched vessels and in the frequency of lymphatic contractions strongly suggests their participation in the pacemaking mechanism of lymphatic pumping [69, 71]. Indeed, T-type Ca^{2+} channels have also been reported to modulate electrical activity in other smooth muscles, such as rabbit urethra and guinea pig detrusor muscle of the urinary bladder, where their inhibition leads to a decrease in action potential frequency [99, 100]. Moreover, a role for T-type Ca^{2+} channels in the pacemaking mechanism of the heart has been demonstrated, as blocking T-type Ca^{2+} channels in the sinoatrial node slows the cardiac diastolic depolarization, also referred to as the "pacemaker potential" [101, 102]. Indeed it has been observed that increases in action potential/contraction frequency evoked by step increases in stretch in rat mesenteric collectors can occur without changes in membrane potential [55]. Thus, it is likely that stretch activates a collection of depolarizing ion conductances, including a T-type Ca^{2+} conductance, that modulate the frequency of action potentials/contractions in lymphatic muscle. Inhibition of one of these conductances, such as T-type Ca^{2+} channels, would lead to slower frequencies, but not necessarily affect the membrane potential. Further investigations are required to determine the specific role of T-type Ca^{2+}channels in the generation of APs leading to lymphatic contractions.

Other ion channels, which could, individually or in combination, play a role in the mechanosensor mechanism are members of the transient receptor potential family (TRP), such as TRPC6, TRPM4, and TRPM7 [103–107]. They have been shown to participate in cardiac automaticity [108] or pacemaking in intestinal interstitial cells of Cajal (ICC), sometimes in concert with ANO1 Cl_{Ca} channels [106, 107]. Importantly, like Cl_{Ca} channels, many TRP channels are permeable to Ca^{2+} and/or have their activity modulated by this ion [105, 109–111]. Although most are expressed in lymphatic vessels [112] (von der Weid, unpublished data), TRP channels have not been investigated in the context of lymphatic pacemaking.

15.6 Pathologies of the Lymphatic Vessels

15.6.1 Lymphedema

The importance of the lymphatic vessels in fluid and macromolecule balance is obvious in a case of lymphatic failure. One of the most common clinical consequences of inadequate lymphatic functioning is *lymphedema*. This affliction is a swelling of the tissues caused by an accumulation of fluid and proteins [113], usually consequent to abnormalities in the regional lymphatic drainage of the extremities, although visceral lymphatic abnormalities can also occur [114]. In contrast to venous edema in which enhanced capillary pressure indirectly stimulates lymph production, lymphedema is caused by a reduction in lymphatic transport. Lymphedema is typically categorized as *primary* (congenital) if the abnormality preventing lymph flow exists in the lymph vessels or lymph nodes, or as *secondary* (acquired) if the disease obstructing or obliterating the lymph conducting pathways began elsewhere [115].

The development of lymphedema can be the result of anatomic problems, including lymphatic hypoplasia and functional insufficiency or absence of lymphatic valves [116, 117]. Indeed, if lymphatic endothelial dysfunction is present, lymphatic muscle dysfunction is likely to follow. Impaired lymphatic drainage fosters the accumulation of protein and cellular metabolites, followed by an increase in tissue colloid osmotic pressure, which leads to water accumulation and increased interstitial hydraulic pressure. Once a chronic state is reached, an increase in the number of fibroblasts, adipocytes, and keratinocytes in the edematous tissues is observed. Macrophages often denote the chronic inflammatory response [118]. Increased collagen deposition, with adipose and connective tissue overgrowth in and around the edematous tissue (usually skin), also occurs, leading to progressive fibrosis [114].

Recent advance in the genetic investigation of primary lymphedemas has permitted the identification of forkhead transcription factor FOXC2 as a candidate gene for lymphedema-distichiasis [119]. FOXC2 is involved in abnormal interactions between lymphatic endothelial cells and pericytes as well as valve defects, which are characteristic of the pathology of lymphedema-distichiasis [120]. Hereditary primary lymphedema (also referred to as Milroy's disease) is attributed to a mutation that inactivates VEGFR-3 tyrosine kinase signaling important mainly in lymphatic vessels [121, 122].

Secondary lymphedema is much more common than the primary form. It develops after disruption or obstruction of lymphatic pathways by surgical, traumatic, inflammatory, and neoplastic disease processes. Edema of the arm after excision of axillary lymph nodes and subsequent irradiation, classical procedures to alleviate breast cancer, is probably the most common cause of lymphedema in developed countries [123]. Although multiple contributing mechanisms can be suggested, lymphoscintigraphy experiments have demonstrated that lymphatic pump failure participates in the lymphatic dysfunction observed in these patients [124].

However, the most common cause of secondary lymphedema is lymphatic filariasis, a major public health problem throughout many regions of the tropics, which affects an estimated 120 million persons worldwide [125]. The disease is caused by several species of filarial nematode, the most common being *Wuchereria bancrofti*. Parasites are transmitted by mosquitos and infective larvae develop into adult worms in afferent lymphatic vessels, causing severe distortion of the lymphatic system. In their life span of several years, adult *Wuchereria* can release millions of larvae into the blood, which often lodge in the lymphatics of the spermatic cord, causing scrotal damage and swelling.

Elephantiasis—a painful, disfiguring swelling of the limbs—is a classic sign of late-stage disease. In addition to these chronic pathologies, infected people experience several episodes of acute inflammatory disease each year, associated with the death of adult worms and infection with opportunistic organisms invading damaged and dysfunctional lymphatics and surrounding tissues. Until recently, understanding of filarial disease was considered to be due to complex interactions

between the parasite, host immune responses, and opportunistic infections. Studies aiming at characterizing the molecular nature of the inflammatory stimuli derived from filarial nematodes have revealed the critical role played by the worms' symbiont *Wolbachia* [126, 127]. Indeed, lipopolysaccharide (LPS)-like molecules released from these intracellular bacteria are responsible for potent pro-inflammatory responses by macrophages in animal models of filarial disease [128]. *Wolbachia* has also been associated with severe inflammatory reactions to filarial chemotherapy, being released into the blood following death of the parasites. Recent studies in animal models even implicate *Wolbachia* in the onset of lymphedema. Taken together, these studies imply a major role for *Wolbachia* in the pathogenesis of filarial disease. It may be possible, through the use of antibiotic therapy, to clear worms of their symbiotic bacteria, with the intent that this approach will prevent the development of filarial pathology [129].

While the precise pathological mechanisms that produce lymphedema during filariasis are not completely understood, symptoms including lymphangitis, dilated lymphatics, and decreased lymphatic contractile function both *in situ* and *ex vivo* point to an inhibition of lymphatic muscle function leading to a loss of lymphatic contractile activity and ultimately the development of lymphedema [130, 131].

15.6.2 Inflammation-Induced Lymphatic Contractile Dysfunction

As eluded above, the low-grade inflammation underlying the lymphedema condition might be a contributing factor in the lymphatic dysfunction. Indeed, collecting lymphatic vessels usually change their contractile behavior upon inflammatory stimulation. These inflammation-induced alterations are characterized first by dilation of the vessel and decrease in contraction frequency. Many studies have been performed to dissect out the role of individual components of the inflammatory soup in lymphatic pumping modulation. Classical inflammatory mediators, such as prostanoids, histamine, or NO, have all been shown to modulate lymphatic pumping and lymph drainage (see reviews [43, 132]). In addition, neuromediators important in immune and inflammatory responses, such as substance P, calcitonin gene-related peptide (CGRP), neuropeptide Y, or vasoactive intestinal polypeptide (VIP), have also been reported to strongly modulate lymphatic vessel contractility [133–136].

In a physiological and homeostatic context, it has been widely demonstrated that endothelium-derived mediators such as NO, prostacyclin, or prostaglandin E_2 inhibit lymphatic contractility [54, 137–145], while on the other hand thromboxane A_2 increases it [140, 143–146]. As these molecules are strongly associated with and upregulated during the inflammatory process, they are very credible candidates to influence lymphatic pumping during inflammation. Indeed, studies demonstrated that the inhibition of mesenteric lymphatic pumping during 2,4,6-trinitrobenzenesulfonic acid (TNBS)-induced ileitis was due to an increase in the production of both NO and prostaglandins [147, 148]. NO was also heavily involved in dilation and suppression of contractions in popliteal lymphatic vessels of mice after oxazolone-induced acute skin inflammation that decreased the lymphatic transport capacity. In this situation, NO was produced by infiltrated $CD11b^+Gr\text{-}1^+$ cells expressing inducible NO synthase (iNOS) [149]. However, dilation and slow pumping of the vessel may be caused by high lymph pressure driven by the upstream edema. In some circumstances, this would favor faster material transport, as demonstrated in isolated vessels from control/healthy animals where increased intraluminal pressure causes dilation and reduced pumping to allow material to quickly travel through the vessel network [150]. The role of pro-inflammatory cytokines has also been experimentally addressed. A study by Hanley et al. [151] reported that in isolated pressurized bovine mesenteric lymphatics interleukin (IL)-1α and 1β caused a significant inhibition of the pressure-dependent increase in lymphatic pumping. Interestingly, this effect occurred within minutes of IL-1 application, precluding a transcription-mediated action. Another study using noninva-

sive near-infrared fluorescence imaging described cessation of murine lymphatic propulsion as early as 4 h following intradermal administration of LPS, IL-1β, TNFα, or IL-6 [26]. Importantly, these effects were noted to be systemic and driven by NO production. In our own hands, we were not able to observe such rapid effects but did observe significant inhibition of pumping in isolated vessels or tonic contraction in cultured lymphatic muscle cells incubated for 24 h with the cytokines IL-1β or TNFα (unpublished observations; [25, 27]). The TNFα-induced decrease in lymphatic pumping requires activation of the NF-κB signaling pathway, upregulation of NOS, and production of NO, which drives lymphatic muscle hyperpolarization via the opening of ATP-sensitive K^+ channels [27].

Metabolic syndrome, which is characterized by chronic subclinical inflammation, also displays impaired intrinsic lymphatic contractility. Prenodal mesenteric collectors in a rat model of metabolic syndrome were significantly smaller in diameter than their control counterparts and had markedly reduced contraction frequency, effectively reducing the intrinsic flow-generating capacity of these vessels by almost 50%. These vessels also exhibited a twofold reduction in their total force production and their myofilaments were significantly less sensitive to Ca^{2+} compared with control myofilament [152].

Collectively, these studies directly indicate that inflammatory changes in the surrounding microenvironment significantly affect the lymphatic contractile behavior and subsequent flow of lymph and highlight the pivotal contribution of the collecting lymphatic vessels in the perpetuation of inflammatory and immune responses.

15.7 Conclusion

Lymphatic pumping is the main driving force behind lymph drainage, which when inappropriate contributes to the development of conditions such as lymphedema and chronic inflammatory diseases. It is initiated in the lymphatic muscle cells comprising the wall of lymphatic collectors by a pacemaker mechanism yet to be fully elucidated. Over the last decades, studies have implicated several different membrane ion channels and a strong dependence on intracellular Ca^{2+} transients in this intrinsic contractile activity. The precise interaction between these elements appears to vary between lymphatic beds and/or animal species. It is likely that given the ability of the lymphatic vessels to respond to changes in lymph pressure, the varying conclusions reached to date are due to differences in experimental design and whether or not stretch was applied to the vessel. Taking these methodological differences into consideration, most of the findings can be reconciled under the premise that several mechanisms are involved in lymphatic pacemaking and that their individual contribution changes depending on the strength or the origin of the mechanical stimulation. Based on current literature, we can suggest that spontaneous contractions occurring in unstimulated vessels heavily rely on the random release of Ca^{2+} from IP_3-sensitive intracellular stores activating ANO1 Cl_{Ca} channels and the generation of STDs, which if large enough allows the triggering of action potentials upon the opening of L-type Ca^{2+} channels. While the involvement of other channels, such as T-type Ca^{2+} channels, in this process can be suggested, there is good consensus as to a primary role for IP_3-sensitive Ca^{2+} stores and Cl_{Ca} channels. When a lymphatic collector is pressurized, the importance of these elements decreases as the increase in wall tension activates a different set of molecular entities that constitute the stretch-induced pacemaker. The molecular elements involved in this stretch-induced activity then provide the depolarization (directly or via intracellular Ca^{2+} changes) necessary for the action potential-induced contraction. Current investigations, testing the role of mechanosensitive TRP channels, should shed some light on this fascinating research area and may lead to the development of therapeutic tools useful to correct lymphatic contractile dysfunction and regulate edema and inflammation as it occurs in lymphedema and chronic inflammatory diseases.

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