Spontaneous transient depolarizations in lymphatic vessels of the guinea pig mesentery: pharmacology and implication for spontaneous contractility

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von der Weid PY, Rahman M, Imtiaz MS, van Helden DF. Spontaneous transient depolarizations in lymphatic vessels of the guinea pig mesentery: pharmacology and implication for spontaneous contractility. Am J Physiol Heart Circ Physiol 295: H1989–H2000, 2008; doi:10.1152/ajpheart.00007.2008.—Guinea pig mesenteric lymphatic vessels exhibit rhythmic constrictions induced by action potential (AP)-like spikes and initiated by entrainment of spontaneous transient depolarizations (STDs). To characterize STDs and the signaling mechanisms responsible for their occurrence, we used intracellular microelectrodes, Ca2+ imaging, and pharmacological agents. In our investigation of the role of intracellular Ca2+ release from Ca2+ stores, we observed that intracellular Ca2+ transients accompanied some STDs, although there were many exceptions where Ca2+ transients occurred without accompanying STDs. STD frequency and amplitude were markedly affected by activators/inhibitors of inositol 1,4,5-trisphosphate receptors (IP3Rs) but not by treatments known to alter Ca2+ release via ryanodine receptors. A role for Ca2+-activated Cl− (ClCa) channels was indicated, as STDs were dependent on the Cl− but not Na+ concentration of the superfusing solution and were inhibited by the ClCa channel blockers niflumic acid (NFA), anthracene 9-carboxylic acid, and 5-nitro-2-(3-phenylpropylamino)benzoic acid but not by the volume-regulated Cl− blocker DIDS. Increases in Cl− frequency and amplitude induced by agonist stimulation were also inhibited by NFA. Nifedipine, the hyperpolarization-activated inward current blocker ZD-7288, and the nonselective cation/store-operated channel blockers SKF-96365, Gd3+, and Ni2+ had no or marginal effects on STD activity. However, nifedipine, 2-aminoethoxydiphenyl borate, NFA, SKF-96365, Gd3+, and Ni2+ altered the occurrence of spontaneous APs. Our findings support a role for Ca2+ release through IP3Rs and a resultant opening of ClCa channels in STD generation and confirm the importance of these events in the initiation of lymphatic spontaneous APs and subsequent contractions. The abolition of spontaneous APs by blockers of other excitatory ion channels suggests a contribution of these conductances to lymphatic pacemaking.

lymphatic pacemaking; membrane potential; calcium transients; inositol 1,4,5-trisphosphate receptors; calcium-activated chloride channels

IN MAMMALS, including humans (23), smooth muscle cells in the wall of collecting lymphatic vessels possess the intrinsic property to contract rhythmically, causing the transient compression of successive chambers that comprise the vessels. This “heart-like” contractile behavior represents the primary mechanism of lymph propulsion and causes the pulsatile, forward movement of lymph. A pacemaker-generated action potential (AP) initiates each transient contraction of the lymphatic smooth muscle (3, 6, 35). Studies on the underlying pacemaker mechanism have primarily been performed in vitro on nonperfused lymphatic vessels from the guinea pig mesentery, where the pacemaker activity initiating smooth muscle APs has been shown to involve spontaneous transient depolarizations (STDs) (53, 56). STDs have also been suggested to participate to pacemaking in sheep mesenteric lymphatic vessels (52). Evidence for the role of STDs in lymphatic pacemaking has arisen through observations that 1) STDs have the same rising phase as the foot of APs in short vessel segments (53); 2) the frequency and amplitude of STDs are increased during stimulation with excitatory agonists, leading to rhythmic APs and contractions (21, 34, 53, 58); and 3) the frequency and amplitude of STDs are decreased in response to agents that slow lymphatic pumping (11, 12, 58, 60, 61). Based on these data, we have suggested that spatial and temporal entrainment of store Ca2+ release underlying lymphatic STDs leads to rhythmic pacemaker potentials (PPs), which then trigger APs, allowing Ca2+ entry through L-type Ca2+ channels and vessel constriction (34, 55, 56).

STDs in lymphatic smooth muscle were considered to arise through Ca2+ release from inositol 1,4,5-trisphosphate (IP3) receptor (IP3R)-mediated Ca2+ stores, as STD activity was abolished by intracellular Ca2+ chelation with BAPTA-AM (53) and inhibition of Ca2+ reuptake into stores by cyclopiazonic acid (CPA) (20). This proposal was also supported by findings that STD activity was enhanced by the application of excitatory agonists known to act via increase in IP3 synthesis and release of Ca2+ from stores, such as norepinephrine, the thromboxane A2 mimic U-46619, histamine, and endothelin (21, 53, 61, 63). The importance of IP3 and Ca2+ store release in the initiation of STDs has been further substantiated in a recent study (34) where a mathematical model based on quantal release of Ca2+ from IP3-sensitive Ca2+ stores and Ca2+ stores interacting as a coupled oscillator was successfully applied to lymphatic pumping. The observation that STDs reversed between −30 and −25 mV, close to the equilibrium potential for Cl−, led to the suggestion that the inward current underlying STDs was generated by Cl− (53). However, to date, pharmacological characterization of STDs has not been thoroughly examined in lymphatic vessels. This was addressed in the present study, which tested the hypothesis that guinea pig

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lymphatic STDs reflect the opening of Ca\(^{2+}\)-activated Cl\(^{-}\) (Cl\(_{Ca}\)) channels following IP\(_{3}\)-R-mediated store Ca\(^{2+}\) release.

**METHODS**

**Tissue preparation.** Guinea pigs (7–15 days of age) of either sex were killed by decapitation during deep anesthesia induced by an inhalation of halothane (5–10% in air). This procedure was approved by the Animal Care and Ethics Committee of the University of Calgary, where the experiments were performed, and conformed to guidelines established by the Canadian Council on Animal Care. The small intestine with its attached mesentery was rapidly dissected and placed in a physiological saline solution (PSS; see below).

Small collecting lymphatic vessels (diameter: 80–230 \(\mu\)m) supplying the jejunum and ileum were dissected together with their associated artery and vein and left intact within the surrounding mesentery. The mesentery was either pinned flat onto the sylgard-coated bottom of a 100-ml organ bath or held flat against the glass coverslip base of a larger 0.5- to 1-ml bath using a fine stainless steel frame with the latter used for the Ca\(^{2+}\) imaging experiments. The pinning procedure appeared to exert only mild physical forces on the lymphatics, tending to flatten the vessels but causing little if any stretch due to the vessels running inside pocket-like regions of the mesentery such that the mesentery absorbed the stretch. In this situation, the vessels showed relatively little spontaneous activity under resting conditions but could readily be stimulated by the application of excitatory agonists. Experiments were made while preparations were continuously superfused with PSS at a flow rate of 3 ml/min in the electrophysiology bath, causing 90% change over time in 7 s and 5 ml/min in the Ca\(^{2+}\) imaging bath. The PSS was heated through a water jacket circuit to a temperature in the organ bath set to 36°C.

**Ca\(^{2+}\) imaging.** Ca\(^{2+}\) imaging experiments were made using a Bio-Rad 1000 confocal laser system (Cambridge, MA) attached to an inverted microscope (Nikon TE200) or Nipkow disk-type confocal microscope attached to a Zeiss IM10 inverted microscope. Tissues were viewed directly through the coverslip that formed the base of the bath with a water-immersion objective (magnification: \(\times 60\) and numerical aperture: 1.2) or an oil objective (magnification: \(\times 20\) and numerical aperture: 0.8). Tissues were loaded by two procedures. The first was to luminally perfuse vessels for 30 min at 35°C with PSS in which the CaCl\(_2\) concentration was reduced to 1 mM and that contained 2 \(\mu\)M Oregon green-AM (Molecular Probes, Eugene, OR). This was followed by a 5-min perfusion using the normal low-Ca\(^{2+}\) (1 mM) saline solution to wash out extra dye in the vessel lumen. Loading of the smooth muscle by this procedure was only successful in endothelium-denuded vessels, a condition that was achieved by briefly passing bubbles of air through the vessel lumen (see Ref. 61). Vasomotion in these vessels occurs independent of the endothelium with endothelial factors only subserving a modulatory role (59). The second method was to externally load the smooth muscle by adding \(-10\) \(\mu\)M Oregon green-AM to the normal PSS for 1 h at room temperature. This was used for loading lymphatic smooth muscle in tissues in which the mesothelium had been largely removed. The Oregon green-loaded lymphatic smooth muscle was excited with light of 488-nm wavelength using an argon ion laser. Emission fluorescence was collected through a 510-nm dichroic mirror and 515-nm bandpass filter. Ca\(^{2+}\) transients were deemed measurable and appropriate for analysis if the associated transient increase in the fluorescence of Oregon green was at least twice the baseline noise. Time-course image frames were inspected, and regions of interest (ROIs) were placed on locations showing an increase in fluorescence related to Ca\(^{2+}\) events. The average fluorescence of all pixels in each ROI was saved as a time-based trace. These traces were divided by the mean fluorescence level during resting baseline for presentation in the figures with associated scale bars.

An in-house MATLAB program (M. S. Imtiaz) was then used to automatically detect and quantify Ca\(^{2+}\) transient events in the ROI traces. The detection method was as follows. First, a 10-point (\(-0.5s\)) moving average smoothing function was applied to each trace, and all local peaks were then detected. A peak was tagged a Ca\(^{2+}\) event if it had 1) a height greater than a height threshold \((H_{th})\) that was 2.5 times SD of noise and 2) a slope \(>3.4(\text{intensity/s})\). The parameter \(H_{th}\) was estimated by placing an ROI on a background region outside the tissue. This was also checked against the baseline noise of ROIs placed on the tissue. An upper limit of 10 \(\times H_{th}\) was used to eliminate AP-related increases in fluorescence. The program plotted all the detected events with height and instantaneous frequency. The output was inspected for errors, and minor adjustments were made if necessary. Several ROIs were analyzed in each tissue, and average frequency and height were calculated.

**Electrophysiology.** Smooth muscle resting membrane potential \((V_m)\) recordings were obtained by impaling cells from the adventitial side of a lymphatic vessel using conventional glass intracellular microelectrodes, with resistances of 150–250 MΩ when filled with 0.5M KCl. Electrophysiological recordings were made using an open circuit to a temperature in the organ bath set to 36°C. This was used for loading lymphatic smooth muscle embedded in the mesentery were cut into short segments (125–350 \(\mu\)m) with fine dissecting scissors. In this situation, electrical activity, even though generated at localized foci within the smooth muscle, produces a similar potential change in all the smooth muscle cells of the segment (53).

Lymphatic smooth muscle impalements were characterized by a sharp drop in potential that settled after 10–15 s to a value typically more negative than \(-45\) mV. Impalements were maintained for \(>5\) min in \(>90\%\) of the cases and up to 3 h optimally. \(V_m\) was corrected at the end of the impalement by subtracting the voltage value obtained after the electrode had been pulled out of the vessels with that of the measured intracellular value. During superfusion with the 10% Ca\(^{2+}\) PSS (see Solutions and drugs), \(V_m\) changes were corrected for the large 35- to 40-mV offset of the voltage baseline. Subthreshold spontaneous depolarizing events \(>1\) mV were considered as STDs, and their activity was assessed by manually measuring their frequency and amplitude. These two measurements can be codependent because STDs encompass a wide spectrum of amplitudes from clearly distinct to smaller events, which merge into baseline noise. Therefore, a change in amplitude is likely to correlate with a change in frequency. STD frequency and amplitude were measured in quiescent segments or between APs over intervals of 15–60 s (depending on the stability of the recording, but typically 30 s). This measurement was compared with that occurring during a period of the same duration while the maximum response to various blockers, agonists, or ion substitution was observed. In experiments where the effects of agonists were studied in the presence of antagonists or inhibitors, agonists were applied first and then at least 20 min later in the presence of the antagonist that had been superfused for at least 10 min. This protocol was usually performed during the same perfusion. However, in some instances, successive impalements were obtained from neighboring cells in the same segment. In preliminary recordings, no significant differences in the responses were found during successive applications of the same agonist at the same concentration.

Lymphatic smooth muscle \(V_m\) and STD activity have been shown to be modulated by factors, such as nitric oxide and prostaglandins, released by the lymphatic endothelium (11, 59, 61). To avoid the potentially confounding endothelial release of these factors in response to the pharmacological agents used in this study, all smooth
muscle recordings were obtained from preparations superfused with
PSS containing 100 μM Nω-nitro-arginine (L-NNA) and 10 μM
indomethacin. These Vm values and the STD activity recorded under
these conditions were used as controls in the present study.

**Solutions and drugs.** The PSS used for the present experiments was
of the following composition (in mM): 2.5 CaCl2, 5 KCl, 2 MgCl2,
120 NaCl, 25 NaHCO3, 1 Na2HPO4, and 11 glucose. pH was main-
tained near 7.4 by constant bubbling with 95% O2-5% CO2. Low-Cl
PSSs (50% Cl- PSS and 10% Cl- PSS) were prepared by replacing
65 or 120 mM Cl- with methanesulphonate and titrating with NaOH
to restore the pH to 7.4. Similarly, low-Na+ PSS was obtained by
replacing 120 mM Na+ with N-methyl-D-glucamine, with the pH
restored to 7.4 with HCl. The following drugs were used: 2-aminoe-
thoxydiphenyl borate (2-APB), anthracene 9-carboxylic acid (9-AC),
caffeine, DIDS, GdCl3, indomethacin, L-NNA, NiCl2, niflumic acid
(NFA), norepinephrine, tetracaine, and thimerosal from Sigma/Al-
drich; D-myoinositol 1,3,5-trisphosphate hexakisacetoxymethyl ester,
2,4,6-tri-O-butyryl [Bt3(1,3,5)IP3-AM], nifedipine, ryanodine, and xesto-
spongin C from Calbiochem; 5-nitro-2-(3-phenylpropylamino)benzoic
acid (NPPB), SKF-96365, and ZD-7288 from Tocris Cookson (Ellis-
ville, MO); N-[2-[(p-bromocinnamylamino)-ethyl]-5-isoquinolinesul-
fonamide-dichloride (H89) and penitrem A from Alexis (San Diego,
CA); and U-46619 from Cayman Chemicals (Ann Arbor, MI). All
drugs were dissolved in DMSO except for caffeine, GdCl3, NiCl2,
norepinephrine, tetracaine, thimerosal, SKF-96365, and ZD-7288
(distilled water), L-NNA (0.1 M HCl), indomethacin and 9-AC (eth-
anol), and penitrem A (methanol) to give 10 mM stock solutions (100
mM for 9-AC), which were then diluted in PSS to achieve the
appropriate concentrations. The final concentration of the vehicles
was <0.1% when one drug was added but could reach higher
concentrations (i.e., 0.2%) when several drugs were used together.
Even at their highest concentrations, vehicles had no effects on
lymphatic Vm and STD activity.

**Statistical analysis.** Data are expressed as means ± SE, with n
being the number of impalements. Typically, only one impalement for
any given experiment was obtained from one vessel, and a maximum
of 2 vessels/animal were used. Statistical significance was assessed
using a two-tailed paired Student’s t-test with P < 0.05 being
considered significant.

**RESULTS**

Vm and/or Ca2+ imaging recordings from smooth muscle in
lymphatic segments revealed the occurrence of STDs, APs, and
Ca2+ transients (Fig. 1, A and B). STDs have long been
considered generated by Ca2+ release from intracellular stores;

![Fig. 1. Action potentials (APs), spontaneous
transient depolarizations (STDs), and associ-
ated spontaneous Ca2+ transients in lymphatic
vessels segments. A: original intracellular mi-
croelectrode membrane potential (Vm) record-
ing showing the electrical activity occurring in
lymphatic smooth muscle. This particular seg-
ment exhibited rhythmical spontaneous APs
with some STDs occurring in between. The
application of nifedipine (10 μM) inhibited
AP activity, unmasking lower amplitude pace-
maker potential (PP) events, which with
longer exposure to nifedipine were reduced to
STDs. Vm values in this trace and all traces in
subsequent figures are indicated on the left
side of the traces. B, left: section of another
lymphatic chamber showing Oregon green-
loaded lymphatic smooth muscle with regions
of interest (ROIs; 1–7) used for data analysis.
Right, Vm recording from the same chamber
(top trace) and simultaneous recordings of
intracellular Ca2+ for the ROIs marked in the
left. The trace labeled “mean” is the average of
traces 1–7. Arrowheads indicate examples of
correlated occurrence of STDs and Ca2+ tran-
sient events. The segment was not spontane-
ously active but showed spontaneous varia-
tions in activity.

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however, the correlation between STDs and Ca$^{2+}$ transients on a one-to-one basis was sporadic (Fig. 1B). In many cases, STDs were recorded without associated Ca$^{2+}$ transients or Ca$^{2+}$ transients occurred without evoking STDs. In contrast, as we have previously reported (34), APs exhibited large associated Ca$^{2+}$ transients. Consistent with previous reports (7, 21, 34, 43), these events occurred as a result of L-type Ca$^{2+}$ channel opening and could be inhibited by nifedipine (Fig. 1A). In the presence of this blocker, subthreshold PPs were revealed, which were themselves then disrupted to unmask unitary STDs (n = 7, Fig. 1A) (see also Ref. 34).

**Store origin of intracellular Ca$^{2+}$ in STD activity.** Previous studies have demonstrated a sensitivity of STDs to BAPTA-AM and CPA (20, 53), consistent with a role for cytosolic Ca$^{2+}$ in the initiation of these events. To assess the origin of the cytosolic Ca$^{2+}$ involved in STD activity, experiments were performed in the presence of known inhibitors of Ca$^{2+}$ release from IP$_3$-sensitive Ca$^{2+}$ stores and ryanodine-sensitive Ca$^{2+}$ stores. Two agents demonstrated as membrane-permeant blockers of IP$_3$Rs, 2-APB (4, 40) and xestospongin C (22), were first tested. 2-APB (25 and 50 μM) markedly decreased STD frequency and amplitude, with significance reached at 50 μM (Fig. 2, A and B). 2-APB (50 μM) abolished the spontaneous intracellular Ca$^{2+}$ transients associated with STDs (n = 3; Fig. 2C). The fact that 2-APB had a larger effect on Ca$^{2+}$ transients than on measured STDs suggests contributions to membrane potential fluctuations by other sources, which have increasing influence when the fluctuations are small. 2-APB also typically caused a small hyperpolarization of Vm at 50 μM (n = 4) and 4 at 25 μM (n = 4). The same trend was observed in the presence of 5 μM xestospongin C, and only the decrease in STD amplitude was significant for the sample size used (n = 4; Fig. 2B). Support for a role for IP$_3$Rs was also provided by the observation that STD frequency and amplitude were significantly increased in the presence of thimerosal, a sulfhydryl reagent described to increase the sensitivity or affinity of the IP$_3$R for IP$_3$ (9, 20, 29) and Bt$_3$(1,3,5)IP$_3$-AM, a membrane-permeant analog of IP$_3$. 

![Fig. 2. Effects of 2-aminoethoxydiphenyl borate (2-APB) and xestospongin C (Xesto C) on STDs and Ca$^{2+}$ transients.](image-url)
Caffeine is also a potent phosphodiesterase activator of RyRs, also described as an inhibitor of stores in STD activity, we examined the effect of caffeine, a Bt3(1,3,5)IP3-AM could be observed 1–2 min after the beginning of the 5-min application and was maximum at −10 min. In contrast, ryanodine, which at the concentration used (30 μM) is known to block ryanodine receptors (RyRs) and hence Ca2+ release from ryanodine-sensitive stores, did not significantly affect STD activity (n = 10; Fig. 4A). To further compare the relative roles of IP3-sensitive and ryanodine-sensitive Ca2+ stores in STD activity, we examined the effect of caffeine, a powerful activator of RyRs, also described as an inhibitor of IP3s (8, 45, 46). Caffeine is also a potent phosphodiesterase inhibitor (see Ref. 18), an action leading to an increase in the intracellular levels of cAMP (39). In our preparation, an increase in cAMP has been previously reported to cause a potent hyperpolarization of the lymphatic smooth muscle and a decrease in STDs (57, 60, 61). To avoid these confounding actions, experiments were performed in the presence of the cAMP-dependent protein kinase inhibitor H89 (10 μM). Under these conditions, caffeine (5 mM) caused an initial 5 ± 1-mV depolarization (n = 14) that was sometimes followed by a hyperpolarization (range: 1–10 mV). The caffeine-induced depolarization was usually associated with an increase in STD activity followed by a decrease (Fig. 4, A and B). The same experiment was repeated in the presence of 50 μM tetracaine to evaluate the role of RyRs on the caffeine-induced response. Under these conditions, caffeine no longer caused an initial increase in STDs, but the depolarization (4 ± 1 mV, n = 6) and subsequent decrease in STD activity were not affected (Fig. 4, A and B).

**Ionic dependence of STDs.** The ionic selectivity of the conductance underlying STD generation was investigated during superfusion with PSS where Cl− or Na+ was substituted with nonpermeant ions (see METHODS). Measurements of STDs at the end of a 2- to 3-min substitution with 10% Cl− PSS demonstrated marked enhancements in STD frequency and amplitude (Fig. 5, A and C). Once Vm values were corrected for offset, low-Cl− PSS-associated depolarizations averaging 7 ± 3 mV (n = 5) were observed. Similar experiments performed in 50% Cl− PSS showed no significant changes in STD frequency (102 ± 8% of control) and amplitude (97 ± 7% of control, n = 8) but a depolarization of 2 ± 1 mV. However, in four occasions where segments exhibited AP-like spikes, the frequency of these events was markedly increased in 50% Cl− PSS. When Vm recordings were obtained during superfusion with low-Na+ PSS, STD frequency and amplitude were not significantly affected (115 ± 14% and 116 ± 8% of control, respectively, n = 5). In this low-Na+ PSS, the frequency of AP-like spikes was not altered (n = 2).

**Involvement of ClCa conductance in STD activity.** The involvement of intracellular Ca2+ and Cl− in the generation of STDs was further investigated using NFA and 9-AC, pharmacological agents known to inhibit ClCa channels. As shown in Fig. 6, the frequency and amplitude of STDs recorded under control conditions were significantly and concentration dependently decreased by NFA. This was not due to changes in Vm, which was only marginally altered [i.e., 3 ± 1 mV at 25 μM (n = 7, P < 0.01), 3 ± 1 mV at 50 μM (n = 9, P < 0.01), and 6 ± 1 mV at 100 μM (n = 7, P < 0.01)]. However, it could have been at least in part due to a direct action on Ca2+ release, as NFA (50 μM) also decreased the frequency and amplitude of spontaneous intracellular Ca2+ transients (80.5 ± 4.1% and

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**Fig. 3.** Role of inositol 1,4,5-trisphosphate (IP3) on STD activity. A and B: intracellular microelectrode Vm recordings showing STD activity under control conditions (top traces) and in the presence of thimerosal (A, bottom trace) and the IP3 analog D-myo-inositol 1,3,5-trisphosphate hexakisacetoxymethyl ester, 2,4,6-tri-O-butyryl [Bt3(1,3,5)IP3-AM; B, bottom trace]. Scale bars in B apply to all traces. C and D: STD frequency and amplitude measured at the maximum effect of the agonists thimerosal (C) and Bt3(1,3,5)IP3-AM (D). Data are expressed as percentages of values obtained during the same impalement before agonist application. Columns are means ± SE with the numbers of experiments indicated in parentheses. *P < 0.05 vs. control (by paired Student’s t-test).
Fig. 4. Effect of ryanodine, tetracaine, and caffeine on the occurrence of STDs. A: summary data of STD frequency and amplitude recorded during the application of ryanodine (30 μM), tetracaine (50 μM), caffeine (5 mM), and caffeine plus tetracaine. Only caffeine had a significant action on STD frequency and amplitude, and this effect was not altered by tetracaine. *P < 0.05 vs. control (by paired Student’s t-test). Columns are means ± SE with the numbers of experiments indicated in parentheses. B: Vm recordings showing the decrease in STD activity in the presence of 5 mM caffeine (top traces) and while ryanodine receptors were blocked by 50 μM tetracaine (bottom traces). H89 (10 μM) was present throughout the caffeine experiments. Scale bars apply to all traces.

55.7 ± 1.3% of control, respectively, n = 3 with 3–5 ROIs/vessel).

STD frequency and amplitude were also significantly reduced by 9-AC (1 mM, n = 7, P < 0.05; Fig. 6B), which concomitantly hyperpolarized lymphatic smooth muscle by 3 ± 1 mV (n = 7, P < 0.01). NPPB, a blocker of both ClCa and volume-regulated Cl− (Clvol) channels, strongly hyperpolarized the lymphatic smooth muscle (13 ± 4 mV at 50 μM, n = 4, P < 0.05) and decreased STD activity (Fig. 6B). In contrast, STD activity was not significantly altered by the classical Clvol channel blocker DIDS (50 and 300 μM; Fig. 6B). Vm was not affected with values of −49 ± 2 mV in control versus −50 ± 3 mV in 300 μM DIDS (n = 4) and −46 ± 0.4 mV in control versus −44 ± 1 mV in 50 μM DIDS (n = 5). The ClCa channel blockers used in this study, in particular NFA, have been also shown to activate large-conductance Ca2+−activated K+ (BKCa) currents. We thus used the selective BKCa blocker penitrem A to assess the possible role of this conductance in STD generation/modulation. We also evaluated the role of BKCa channels in NFA-induced STD inhibition. In the presence of 1 μM penitrem A, STD frequency and amplitude were not significantly affected (92 ± 10% and 99 ± 5% of control, respectively, n = 8) and were still markedly decreased by 50 μM NFA (41 ± 15% and 81 ± 5%, respectively, n = 6, P < 0.05). NFA caused a hyperpolarization paralleling that observed without penitrem A (2 ± 1 mV, n = 7).

Pharmacology of agonist-induced STDs. STD activity has been shown to increase in response to the application of agonists, such as norepinephrine and U-46619 (53, 61) as well as thimerosal (see above). NFA was able to limit these increases, reducing STD activity to values below control in the absence of the activators (Fig. 7). NFA treatment also abolished the depolarizations induced by these agonists with Vm repolarizing close to values before agonist application (Fig. 7, A and B). Similar results were obtained in the presence of 9-AC (1 mM, n = 2; data not shown).

Role of other excitatory conductances on STD activity. STD activity was examined in the presence of blockers of other depolarizing conductances. Neither STD frequency nor amplitude was significantly altered in the presence of the L-type Ca2+ channel blocker nifedipine (1 μM) or the T-type Ca2+ channel blocker Ni2+ (30 μM, n = 3, and 500 μM; Fig. 8). The nonselective cation channel (NSCC) blocker SKF-96365 (10 μM) did not alter STD frequency but significantly decreased STD amplitude, whereas Gd3+ (50 μM), another NSCC blocker, slightly but significantly increased the frequency of STDs without altering their amplitudes (Fig. 8). The involvement of STDs of the hyperpolarization-activated depolarizing current (Ih), which has been suggested to play a pacemaker role in sheep mesenteric lymphatics (32, 33, 42), was assessed using the selective blocker ZD-7822. ZD-7822 (1 μM) neither altered STD frequency nor amplitude (Fig. 8). Despite their modest or insignificant effects on STD activity, 1 μM ZD-7822, 50 μM Gd3+, and 10 μM SKF-96365 depolarized smooth muscle Vm by 4–5 mV (n = 5–6, P < 0.05). However, Vm was not significantly affected by 1 μM nifedipine or 500 μM Ni2+ (n = 4 for both).
Effects of excitatory ion channel inhibition on spontaneous APs. When spontaneous AP-like spikes occurred in the short lymphatic vessel segments, they could be inhibited by NFA (50–100 μM, n = 3; Fig. 9A) and NPPB (50–100 μM, n = 3; data not shown). These agents further inhibited the underlying PPs and ultimately STDs. In line with their action to block STDs, 2-APB (50 μM, n = 4) and caffeine (5 mM, n = 2) also abolished spontaneous APs, whereas tetracaine (50 μM, n = 3) and ryanodine (30 μM, n = 3) had no effect on this activity. Importantly, Gd3+ (10 and 50 μM), SKF-96365 (10 μM), and Ni2+ (30 μM) also abolished spontaneous APs, revealing PPs and then STDs (n = 2–4; Fig. 9). The traces shown in Fig. 9 also exemplify the wide range of spontaneous spike-like AP patterns that were observed in lymphatic vessel segments. Irrespective of this variability, the blockers consistently abolished spontaneous APs.

DISCUSSION

In lymphatic vessels of the guinea pig mesentery, intracellular microelectrode recordings from short vessel segments or from small lymphatic chambers in intact vessels have revealed the existence of STDs (53). These depolarizations are generated in the lymphatic smooth muscle syncytium, and entrainment of this activity provides a likely basis for the initiation of APs and consequent rhythmical transient contractions (53, 55).

In lymphatic smooth muscle, intracellular Ca2+ has been demonstrated to be critical to lymphatic constrictions and to STDs, with both activities being abolished in the presence of the Ca2+ chelator BAPTA-AM and the sarco(endo)plasmic reticulum Ca2+-ATPase inhibitor CPA (5, 20, 53). The pharmacological data from the present study and these previous studies strongly indicate that STDs are the consequence of Ca2+ release events from intracellular Ca2+ stores. However, despite this, simultaneous electrophysiological and Ca2+ imaging recordings showed inconsistent correlation between Ca2+ transients and STDs, and we cannot be certain that Ca2+ transients are causative or coincidental (Fig. 1B). A contributing factor to the weak correlation results from our present experimental method, where STDs were recorded from the upper side of vessel segments and Ca2+ events from the underside of a limited region of vessel segments. Nevertheless, Ca2+ transients were expected to cause depolarization, as vessel segments were electrically short; however, relatively few were associated with STDs. The weak correlation was also exacerbated by STDs that occurred without corresponding events on Ca2+ imaging traces. This could happen if the corresponding Ca2+ transients were initiated outside the field of view. It may also be that the Ca2+ release that generates STDs dominantly occurs in subplasmalemmal microdomains, which are not adequately sampled by conventional Ca2+ im-
aging. Indeed, our previously reported finding (34) presents evidence of small but measurable global increases in the cytosolic Ca\(^{2+}\) concentration that correlated with the summations of STDs underlying subthreshold pacemaker activity. This indicates that STDs are associated with increases in the cytosolic Ca\(^{2+}\) concentration but that the Ca\(^{2+}\) signal underlying individual STDs is weak when measured in the bulk cytosol. In contrast, the existence of Ca\(^{2+}\) transients without corresponding changes in \(V_m\) suggests that many of these events occur in cells/cellular regions without appropriate follow-on mechanisms (e.g., Ca\(^{2+}\)-activated excitatory channels).

Pharmacological investigations made into the role of Ca\(^{2+}\) stores support the hypothesis that Ca\(^{2+}\) is preferentially released through IP\(_3\)Rs. These conclusions are based on the inhibitory action of the IP\(_3\)R blockers 2-APB and xestospongin C on STDs and Ca\(^{2+}\) transients and on the stimulatory actions of an IP\(_3\) analog and agonists known to increase the synthesis of IP\(_3\). 2-APB has been also shown to inhibit store-operated Ca\(^{2+}\) entry (10, 19, 25, 40, 49), although this action has mainly been observed in cells from hematopoietic or immune lineages and not in smooth muscle. Xestospongin C has been reported to act on voltage-activated Ca\(^{2+}\) currents in guinea pig intestinal smooth muscle (44), but this is unlikely to have been a factor in the present study as STD activity was not affected by inhibition of voltage-activated Ca\(^{2+}\) channels by nifedipine or Ni\(^{2+}\) (see Fig. 8).

RyRs did not play a significant role in STD generation, as neither tetracaine, a membrane-permeant, reversible blocker of cardiac (26), skeletal (47, 62), and smooth (13, 41) muscle RyRs, nor ryanodine had significant effects on STD activity. Consistent with these findings, ryanodine has been shown to have no effect on vasmotion in lymphatic vessels from the guinea pig mesentery (63). Caffeine, applied in the presence of the cAMP-dependent protein kinase inhibitor H89 to block caffeine-associated inhibition of phosphodiesterase and the production of cAMP, inhibited STDs. The action of caffeine was maintained in the presence of the RyR blocker tetracaine, which is consistent with caffeine blocking IP\(_3\)-mediated Ca\(^{2+}\) liberation (45) but not activating RyRs (8). This finding is reminiscent of results obtained from rabbit urethra and guinea pig mesenteric vein segments, where STDs displayed in these preparations were inhibited by caffeine (28, 54).

Electrical activity similar to lymphatic STDs or their underlying spontaneous transient inward currents have been recorded in many vascular and nonvascular smooth muscles and have been suggested to result from transient intracellular store Ca\(^{2+}\) release events (see Ref. 58). These transient Ca\(^{2+}\) increases stimulate Ca\(^{2+}\)-activated inward currents, which in most cases have been demonstrated to be carried by Cl\(^-\) (i.e., Cl\(_{Ca}\) channels). Our present findings indicate that Cl\(^-\) is the major permeant ion underlying lymphatic STDs. The primary evidence is that STD amplitude is enhanced in 10% Cl\(^-\) PSS solution but unchanged in low-Na\(^+\) PSS solution. Although an increase in STD activity was expected as decreasing Cl\(^-\) concentration in the bath solution should move the Cl\(^-\) equilibrium potential to less-polarized values, the effect was quite small and not significant for 50% Cl\(^-\) PSS. This could be a consequence of the reduction in the intracellular concentration of Cl\(^-\) when extracellular Cl\(^-\) concentration is decreased, as shown in the guinea pig vas deferens (1). Therefore, if STDs are due to movements of Cl\(^-\) across the cell membrane, then shifts in reversal potential may be less than predicted (54).

Pharmacological inhibitors known to block Cl\(^-\) channels [i.e., the Cl\(_{Ca}\) channel blockers 9-AC and NFA and the dual Cl\(_{Ca}/Cl_{vol}\) channel blocker NPPB (24, 38)] strongly decreased spontaneous and agonist-evoked STDs. By comparison, DIDS,
a classical blocker of Clvol channels in vascular myocytes (see Refs. 24 and 38) was without effect on STDs. However, the NFA data and possibly those for 9-AC and NPPB are confounded in that NFA also had an inhibitory effect on spontaneous Ca\(^{2+}\)/H\(^{\text{11001}}\) transients. This may be due to an action on sarcoplasmic reticulum (SR) Cl\(^{-}\) channels that are known to be present in the SR of skeletal (36) and smooth muscle (48) and hence may be present in the lymphatic SR. Therefore, while the pharmacological data are inconclusive as a test for ClCa channels, the findings are consistent with the inhibitory actions reported in studies on guinea pig prostatic smooth muscle, where NFA and 9-AC but not DIDS inhibited STDs (37). In contrast, spontaneous transient inward currents were inhibited by DIDS in portal vein smooth muscle cells (31). STD-like events have also been shown to be inhibited by 9-AC in smooth muscle cells isolated from sheep mesenteric lymphatics (52).

NFA has been described as the most potent blocker of ClCa channels in smooth muscle available so far (24, 38). However, in contrast to its inhibitory effect on spontaneous Ca\(^{2+}\)/H\(^{\text{11001}}\) transients reported in the present study, it has been shown to stimulate Ca\(^{2+}\)/H\(^{\text{11001}}\) release from intracellular stores (17) and to activate BKCa channels (24, 38). During superfusion with NFA, lymphatic smooth muscle developed a small hyperpolarization that could either be attributed to inhibition of ClCa activity or to enhancement of BKCa activity. BKCa activity did not obviously subserve a role, as blockade of BKCa channels with penitrem A did not affect STDs or the ability of NFA to block them. The hyperpolarization observed during NFA treatment was still present in penitrem A and may be specific for ClCa channels, as it was also observed with 9-AC and NPPB but not upon inhibition of Clvol channels with DIDS. Hashitani and Edwards (27) also noticed a NFA-induced hyperpolarization in the guinea pig urethra and reached a similar conclusion.

Other inward currents including T- and L-type Ca\(^{2+}\)/H\(^{\text{11001}}\) currents and a hyperpolarization-activated inward current, resembling sinoatrial node \(I_f\), have been suggested to participate to lymphatic pacemaking (32, 33, 42). Using pharmacological blockers of these currents, we showed that they were not responsible for STD activity, as nifedipine, Ni\(^{2+}\) (50 μM), and Gd\(^{3+}\) (500 μM) on STD frequency and amplitude. Data are expressed as percentages of values obtained during the same impalement before the application of blockers. Columns are means ± SE with the numbers of experiments indicated in parentheses. \(* P < 0.05\) vs. control (by paired Student’s \(t\)-test).

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**Fig. 8.** Effect of blockers of excitatory channels on STDs. Summary data show the effects of nifedipine (1 μM), ZD-7922 (1 μM), SKF-96365 (10 μM), Gd\(^{3+}\) (50 μM), and Ni\(^{2+}\) (500 μM) on STD frequency and amplitude. Data are expressed as percentages of values obtained during the same impalement before the application of blockers. Columns are means ± SE with the numbers of experiments indicated in parentheses. \(* P < 0.05\) vs. control (by paired Student’s \(t\)-test).
L-type Ca\(^{2+}\) channel opening and the subsequent lymphatic vessel constriction are likely to be activated by entrainment of STD activity (34, 53). Our data obtained with the L-type Ca\(^{2+}\) channel blocker nifedipine are consistent with this hypothesis, as nifedipine abolished APs and underlying PPs, leaving mostly unitary STDs (see also Ref. 34). Inhibition of spontaneous APs and vessel segment constrictions by agents that inhibit STDs (i.e., NFA and 2-APB) is also consistent with the hypothesis and the reported inhibition of spike-like activity by 9-AC and 2-APB in sheep mesenteric lymphatics (7). Although they have no or only marginal effects on STDs, Ni\(^{2+}\), SKF-96365, and Gd\(^{3+}\) inhibited the generation of spontaneous APs, suggesting a role for other excitatory cation channels in AP generation and/or STD entrainment. Ni\(^{2+}\) has been reported to affect T-type and L-type Ca\(^{2+}\) channels, NSCCs, and the Na\(^{+}/Ca^{2+}\) exchanger (14). At the concentration of 30 \(\mu M\) used in our study, Ni\(^{2+}\) should be effective in inhibiting NSCC and all \(\alpha_1\)-subunits of T-type Ca\(^{2+}\) channels but should not affect the Na\(^{+}/Ca^{2+}\) exchanger, where millimolar concentrations of Ni\(^{2+}\) are required (30). The Ni\(^{2+}\)-induced abolition of spontaneous APs could then be attributed to its action of blocking T-type Ca\(^{2+}\) channels or NSCCs. It should also be noted that voltage-activated channels with characteristics of T-type Ca\(^{2+}\) channels have been identified in mesenteric lymphatic smooth muscle of the sheep (15, 32). Their inhibition by 100 \(\mu M\) Ni\(^{2+}\) led to slowing of the constriction frequency but not the abolition of vessel constrictions. Importantly, the inhibition of spontaneous APs by SKF-96365 and Gd\(^{3+}\) reported in the present study suggest a role for NSCCs. Therefore, it is probable that both T-type Ca\(^{2+}\) channels and NSCCs contribute to lymphatic pacemaking, but the identity of these channels needs further investigation.

In conclusion, our data support the proposal that STDs in guinea pig mesenteric lymphatic smooth muscle arise through spontaneous or agonist-induced release of Ca\(^{2+}\) from IP\(_3\)-sensitive Ca\(^{2+}\) stores, which activates Cl\(_{Ca}\) channels. Entrainment of STDs leads to the generation of PPs, which, when superthreshold, activate L-type Ca\(^{2+}\) channel-mediated APs, causing phasic constriction of lymphatic chambers. Although blockers of other ion channels (i.e., T-type Ca\(^{2+}\) channels or NSCCs) had no major effect on STD activity, their actions to inhibit spontaneous APs suggests an involvement in lymphatic pacemaking, which is thus proposed to be a complex process involving the opening of Cl\(_{Ca}\) channels as the initial electrical event, with other conductances playing a role. Modulation of pacemaking most certainly also depends on hyperpolarizing currents generated by voltage-activated or Ca\(^{2+}\)-dependent K\(^+\) conductances (2, 7, 16), which have only been partially considered in the present study.

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tetramerization from skeletal muscle junctional sarcoplasmic reticulum vesicles.  


