Cyclopiazonic acid decreases spontaneous transient depolarizations in guinea pig mesenteric lymphatic vessels in endothelium-dependent and -independent manners

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1Mucosal Inflammation and Smooth Muscle Research Groups, Department of Physiology and Biophysics, Faculty of Medicine, University of Calgary, Alberta, Canada T2N 4N1; and 2Neuroscience Group, School of Biomedical Sciences, Faculty of Health, University of Newcastle, Callaghan, New South Wales, Australia 2308

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Ferrusi, Ilia, Jun Zhao, Dirk van Helden, and Pierre-Yves von der Weid. Cyclopiazonic acid decreases spontaneous transient depolarizations in guinea pig mesenteric lymphatic vessels in endothelium-dependent and -independent manners. Am J Physiol Heart Circ Physiol 286: H2287–H2295, 2004. First published February 19, 2004; 10.1152/ajpheart.00739.2003.—Guinea pig mesenteric lymphatic vessels exhibit vasomotion through a pacemaker mechanism that involves intracellular Ca2+ release and resultant spontaneous transient depolarizations (STDs) of the smooth muscle membrane potential. This study presents a detailed characterization of the effects of cyclopiazonic acid (CPA) on this pacemaker activity. Microelectrode recordings from smooth muscle in vessel segments revealed that application of CPA (1–10 μM) caused a hyperpolarization accompanied by a decrease in the frequency and amplitude of STDs. The CPA-induced hyperpolarization was abolished after destruction of the endothelium and in the presence of Nω-nitro-arginine (100 μM) or 1H-[1,2,4]oxadiazolol-[4,3-a]quinoxaline-1-one (10 μM), which suggests a contribution of endothelium-derived nitric oxide (EDNO) in this response. In the absence of EDNO-induced effects, CPA decreased the frequency and amplitude of STDs recorded before and in the presence of the thromboxane A2 mimetic U-46619, norepinephrine, or thimerosal. CPA abolished U-46619-induced vasomotion as determined by measurement of constriction-associated intracellular Ca2+ concentration using the ratiometric Ca2+ indicator fura-2. The endothelial actions of CPA were compared with those of ACh, which is known to cause EDNO release in this preparation. Although CPA and ACh both increased endothelial intracellular Ca2+ concentration and depolarized the membrane potential, the kinetics of action for both parameters were markedly slower for CPA than ACh. These results suggest that CPA first hyperpolarizes the lymphatic smooth muscle and decreases STD frequency and amplitude through endothelial release of EDNO, and second, consistent with the action of CPA to inhibit sarcoplasmic reticulum Ca2+-ATPase and deplete Ca2+ stores, it further reduces STD activity. Inhibition of the lymphatic smooth muscle pacemaker mechanism is thought to abolish agonist-induced vasomotion.

pumping; nitric oxide; smooth muscle; lymphatic vasomotion

METHODS

Tissue preparation. Guinea pigs (7–15 days of age) of either sex were killed by decapitation during deep anesthesia consequent to inhalation of halothane (5–10% in air). This procedure has been approved by the University of Calgary Animal Care and Ethics Committee and conforms to the guidelines established by the Canadian Council on Animal Care. The small intestine with attached mesentery was rapidly dissected out and placed in a physiological solution.

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saline solution (PSS) of the following composition (mM): 2.5 CaCl$_2$, 5 KCl, 2 MgCl$_2$, 120 NaCl, 25 NaHCO$_3$, 1 NaH$_2$PO$_4$, and 11 glucose. The pH was maintained at 7.4 by constant bubbling with 95% O$_2$-5% CO$_2$.

Small collecting lymphatic vessels (diameter, 80–230 μm) that supply the jejunum and ileum were dissected together with their associated artery and vein and were left intact within the surrounding mesentery. The mesentery was used to pin out the tissue onto the Sylgard-coated bottom of a small organ bath (volume, 100 μl), and the preparation was mounted on the stage of an inverted microscope (Twinkle; Nikon). The pinning procedure appeared to exert only mild physical forces on the lymphatics; it tended to flatten the vessels but caused little if any stretch due to the vessels running inside pocketlike regions of the mesentery such that the mesentery absorbed the stretch. Such unperfused vessels showed relatively little spontaneous activity under resting conditions but could readily be stimulated by vessel perfusion or application of excitatory agonists. Experiments were performed with tissues continuously superfused with PSS heated to 36°C at a flow rate of 3 ml/min, which caused 90% changeover in <7 s.

**Electrophysiology.** Resting membrane potential was measured using conventional glass intracellular microelectrodes with resistances of 150–250 MΩ when filled with 0.5 M KCl. Electrodes were connected to an amplifier (Intra 767; World Precision Instruments; Sarasota, FL) through an Ag-AgCl half cell. Resting membrane potential was monitored on a digital oscilloscope (VC6525; Hitachi) and simultaneously recorded on a computer via an analog-to-digital converter (PowerLab/4SP, AD Instrument; Mountain View, CA). Intracellular recordings were obtained by impaling smooth muscle cells from the adventitial side of the vessels. Vessels were cut into short segments (125–350 μm) with fine dissecting scissors to ensure simplified electrical properties of the smooth muscle. In this situation, electrical activity, although generated at localized foci within the smooth muscle, produces a similar potential change in all the smooth muscle cells of the segment (48). Endothelial membrane potential recordings were obtained either by deeper penetration of the microelectrode into the vessel or by impaling the first layer of cells in a vessel that has been carefully cut open with the intima then facing up. As described in a previous study (56), endothelial cells are more polarized than smooth muscle cells (resting membrane potential, ~72 vs. ~55 mV) and have a characteristically different response to ACh. These two criteria were used to discriminate between endothelial and smooth muscle intracellular recordings.

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 ≥3 h optimally. CPA was added to the PSS that superfused the preparation either for short (1 min) or longer (4–10 min) durations. In experiments where the effects of CPA were assessed in the presence of inhibitors [NOS-nitro-l-arginine (l-NNA), 1H-[1,2,4]oxadiazolo-[4,3-a]quinoxaline-l-one (ODQ), and glibenclamide], CPA was applied first as a control and then at least 20 min later in the presence of the antagonist, which had been superfused for at least 10 min. This protocol was usually performed during the same impalement. However, in some instances, successive impalements were obtained from neighboring cells in the same segment. There was no significant difference in the responses for repeated treatments of CPA applied at the same concentration 20 min apart.

STD activity was assessed by measuring the frequency and amplitude of events >1 mV in size. STD frequency and amplitude were measured for 15–60 s (depending on the stability of the recording, but typically 30 s) before application of CPA (or other agonists: U-46619, norepinephrine, thimerosal) and were then compared with those occurring for a period of the same duration during the time period when response to the agonist was maximal.

**Functional removal of endothelium.** The lymphatic endothelium was destroyed in vitro after a procedure that has been previously described (11, 12). Briefly, a fine glass micropipette was inserted into the lumen of a previously cut vessel. The micropipette, which was connected to an infusion pump via Teflon tubing, was used to luminally perfuse the vessel with PSS in the direction of the valves. This procedure induced rhythmic constrictions of the vessel. To functionally destroy the endothelium, small air bubbles were passed in repeated streams (5–6 times for 5–10 s; rate, 3–5 μl/min) through the vessel lumen via this micropipette. The success of the endothelial destruction was confirmed by applying ACh (10 μM) before sodium nitroprusside (100 μM) in the superfusion solution while the vessel lumen was perfused. An absence of an ACh-induced decrease in functional STD activity in endothelium-intact vessels and a decrease in contractions to sodium nitroprusside used as confirmation of the success of the procedure. Endothelial destruction based on this testing procedure proved successful in ~50% of treated vessels. The use of sodium nitroprusside was necessary, as it has been shown that 40% of guinea pig mesenteric lymphatic vessels with intact endothelium exhibit a high basal production of NO and hence do not respond in any way to either ACh or sodium nitroprusside (54). Loss of function of the endothelium was further confirmed during the electrophysiological experiments by the absence of endothelium-derived hyperpolarization and an inability to significantly change STD activity in response to 10 μM ACh. Membrane potential responses to ACh are very reliable, as they occur in >95% of the cells with functional endothelium.

**Ratiometric measurement of [Ca$^{2+}$].** Experiments involving measurement of [Ca$^{2+}$]$_i$ changes in the lymphatic smooth muscle or endothelium were performed using the calcium-sensing fluorescent dye fura-2. The smooth muscle of lymphatic vessels was loaded by luminally perfusing endothelium-denuded vessels at room temperature with the membrane permeant fura-2 acetoxymethyl ester (AM, 2 μM) and pluronic acid (0.2% wt/vol) for 30 min. The same procedure was used in endothelium-intact vessels to specifically load the endothelium. After this, the vessel was perfused with control solution for 10 min to wash out extraneous dye. The vessel was then left for an equilibration time of at least 20 min to allow the intracellular esterases to cleave fura-2 AM (14). The mesentery was hooked on small pins glued to the outside of a metal frame (1 × 1 cm) such that the frame positioned the mesentery and associated lymphatic vessels flat against the glass coverslip that formed the bottom of the organ bath (volume, 0.5 ml). The tissue was superfused with heated (34–36°C) PSS at a rate of 6 ml/min, and regions of individual vessel chambers (lymphangions) were viewed by an inverted microscope (Zeiss Axiosvert 10) using a ×40 oil-immersion objective (numerical aperture, 1.3). Ratiometric experiments were made photometrically on vessel chambers. Each such measurement incorporated the response from multiple endothelial or smooth muscle cells in endothelium-intact or -denuded vessels, respectively.

Ratiometric fura-2 measurements were made with vessel chambers alternatively illuminated by a xenon lamp at 340- and 380-nm wavelengths for durations of 50 ms for each wavelength with a 50-ms interval between each exposure. This cycle was repeated at a frequency of ~5 Hz. Emission light was passed through a dichroic mirror (490 nm) and a band-pass filter (510 nm) and collected by a photomultiplier; the output response was digitized and captured by computer. Experiments were performed in the absence of luminal perfusion to minimize movement. These movements were then sufficiently small to allow the fura-2-loaded smooth muscle or endothelium to remain in the focal plane. Such movement could in some cases cause the fluorescence at the 380-nm wavelength (F$_{380}$) to increase instead of decrease. This most likely occurred due to more dye-loaded tissue moving into focus and causing a net increase in F$_{380}$ fluorescence. This is accounted for by a proportional change in the signal for fluorescence at the 340-nm wavelength (F$_{340}$) so that the ratio cancels out such movement artifact if the ratios are obtained at a time interval of sufficiently short duration during which movement is small. Independent measurement of constrictions suggests that this was upheld during most of the constriction except for the period of maximum

**References:**

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onset when there was some error. Vessel chambers typically
constricted to peak amplitude in <0.5 s and then decayed relatively
slowly to baseline over the next 2–3 s. The protocol used to measure
the $F_{340}/F_{380}$ ratios incurred a maximum interval between ratios of
100 ms during which there could be up to $\sim 25\%$ movement during
the maximum-onset phase of constriction but less than $\sim 5\%$ during
the decay phase. Thus the ratios would only be substantially distorted
during the maximum-onset phase of constriction (i.e., during the
first 0.5 s of the Ca$^{2+}$ transient).

Chemicals and drugs. ACh, CPA, glibenclamide, L-NNA, norepi-
nephrine, sodium nitroprusside, and thimerosal were all purchased
from Sigma; fura-2 AM was from Molecular Probes (Eugene, OR),
ODQ was from Alexis (San Diego, CA), and U-46619 was from
Cayman Chemicals (Ann Arbor, MI). CPA, fura-2 AM, gliben-
clamide, ODQ, and U-46619 were dissolved in dimethylsulfoxide;
1-NNA was dissolved in 0.1 M HCl, and the remainder were dissolved
in distilled water to yield 10 mM stock solutions. After dilution of
the drugs to their final concentrations in physiological saline, the diluted
vehicle had no effects on tissue activity.

Data analysis. Experimental data are expressed as means $\pm$ SE.
Statistical significance was assessed using a two-tailed, paired Stu-
dent’s $t$-test (unless specified otherwise in the text) with $P < 0.05$
being considered significant.

RESULTS

Effects of CPA on STDs and smooth muscle membrane
potentials. CPA (1–10 $\mu$M) caused a concentration-dependent
hyperpolarization of the smooth muscle (Fig. 1, A and B). The
hyperpolarization reached a peak value of 7.9 $\pm$ 1.3 mV at 10
$\mu$M CPA from a resting value of $-50.4 \pm 1.0$ mV ($n = 21$). During
the hyperpolarization, the activity of STDs, which occurred in $\sim 95\%$ of the lymphatic smooth muscle recordings
(see Refs. 48, 54) was also reduced in a concentration-depen-
dent manner (Fig. 1, C and D). At the peak of the hyperpolar-
ization induced by CPA (10 $\mu$M), STD frequency was signif-
icantly reduced to 48 $\pm$ 7% of control and STD amplitude
decreased to 76 $\pm$ 4% of control ($n = 21; P < 0.01$). In five
recordings, the CPA-induced hyperpolarization was preceded
by a small depolarization of 1–4 mV and an associated in-
crease in STD frequency and amplitude (143 $\pm$ 24 and 134 $\pm$
11% of control, respectively). Alterations in STD activity and
hyperpolarization persisted for 3–5 min after the CPA had been
washed out before returning to control values.

Role of endothelium and EDNO in CPA-induced hyperpo-
larization. Studies on blood vessels have shown that CPA
induces the release of EDNO from endothelial cells, which
causes vasodilation (33, 43, 61). Therefore, as EDNO is known
to modulate lymphatic smooth muscle membrane potential (54, 57),
we investigated the roles of lymphatic endothelium and
EDNO in the hyperpolarization in response to CPA. In the
first set of experiments, the response of the smooth muscle mem-
brane potential to CPA was evaluated in endothelium-denuded
lymphatic vessels. In this situation, the known endothelium-
dependent hyperpolarization in response to ACh (54) was
abolished (Fig. 2A). Application of CPA (10 $\mu$M for $>5$ min)
caus ed a small depolarization of 2.0 $\pm$ 0.8 mV, whereas the

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure1.png}
\caption{Effects of cyclopiazonic acid (CPA) on lymphatic smooth muscle membrane potential. A: intracellular microelectrode recordings from two different preparations in response to 10 $\mu$M CPA applied for the duration indicated by horizontal bars. CPA hyperpolarized the smooth muscle and inhibited spontaneous action potentials (large upward deflections, top) and spontaneous transient depolarizations (STDs; small upward deflections, top and bottom). B: concentration-dependent relationship of the CPA-induced hyperpolarization ($n = 5$–21). C: STD activity recorded before (see also A, bottom) and during inhibition by CPA (top) are compared on an expanded time scale. D: concentration-dependent relationship of the decreases in frequency and amplitude of STDs measured at the peak of CPA-induced hyperpolarization expressed as a percentage of the values obtained during the same impalement before CPA application ($n = 5$–21). Columns are means $\pm$ SE. *$P < 0.05$ vs. control (paired Student’s $t$-test). Resting membrane potential values in this and subsequent figures are indicated left of traces. [CPA], CPA concentration.}
\end{figure}
hyperpolarization was reduced to 1.0 ± 1.1 mV (n = 6; P = 0.34; Fig. 2A).

In the second set of experiments, lymphatic vessels were superfused with L-NNa (100 μM) to inhibit the synthesis of EDNO or ODQ (10 μM) and block the production of cGMP. Again, success of the treatments was attested by the inhibition of ACh-induced hyperpolarization (data not shown). In the presence of L-NNa, the hyperpolarization induced by 10 μM CPA was no longer significant (e.g., 2.2 ± 1.1 mV; n = 10; P = 0.08). It was preceded by a depolarization of 8.3 ± 2.4 mV (n = 10; Fig. 2B). In one of the 10 preparations, the CPA-induced responses were not significantly affected by the L-NNa treatment. In the presence of ODQ (10 μM), the hyperpolarization induced by 10 μM CPA was inhibited to nonsignificant levels (0.9 ± 2.8 mV; n = 7; P = 0.2) thus unmasking a depolarization of 2.1 ± 0.6 mV (Fig. 2C). The same finding was also made when CPA was applied in the presence of glibenclamide (10 μM) with the hyperpolarization again nonsignificant (1.5 ± 1.0 mV) and the response then dominated by a depolarization of 2.5 ± 0.9 mV (n = 4; data not shown).

Endothelium-independent effects of CPA on agonist-evoked STDs. The effects of CPA were also investigated during agonist-induced enhancement of STD activity. As described in the previous section, these experiments were performed under conditions where the EDNO contribution was minimized. Experiments were first performed using the thromboxane A2 mimetic U-46619. At a concentration of 0.1 μM, U-46619 increased STD frequency and amplitude (n = 6; Fig. 3, A and D; see also Ref. 57). U-46619 (0.25 μM) caused increased STD activity, a large transient depolarization, and the nearrhythmic generation of action potentials (Fig. 4A). The U-46619-induced increase in STD activity was significantly reduced below control levels (P < 0.05) during application of 10 μM CPA to vessel segments (n = 6; Fig. 3, A and D). Experiments were also performed in the presence of norepinephrine, which enhances STD activity through activation of α-adrenoceptors (Fig. 3D; Ref. 48). Application of 10 μM CPA to vessel segments exposed to 10–50 nM norepinephrine reduced STD frequency and amplitude (n = 4; P < 0.05; Fig. 3, B and D). The third activator used was thimerosal, an agent reported to sensitize inositol 1,4,5-trisphosphate (IP3) receptors and lead to enhancement of IP3-induced Ca2+ release (4, 5, 18). Application of 1 μM thimerosal increased STD frequency and amplitude to 128 ± 12 and 148 ± 35% of control, respectively (n = 5). Application of 10 μM CPA to vessel segments exposed to 1 μM thimerosal reduced STD frequency and amplitude to values below control (n = 5; Fig. 3, C and D).
The membrane potential usually depolarized by 2–5 mV in the presence of any of these agonists and by another 4–10 mV upon CPA application.

**Endothelium-independent effects of CPA on smooth muscle \([Ca^{2+}]_{i}\).** CPA-induced changes in \([Ca^{2+}]_{i}\), were investigated in endothelium-denuded mesenteric lymphatic vessels using the ratiometric \(Ca^{2+}\) indicator fura-2. Application of 10 \(\mu M\) CPA transiently increased the basal \([Ca^{2+}]_{i}\), to 116 ± 1% (\(n = 3\)); this finding is consistent with that previously reported (60). Transient spikelike increases in \([Ca^{2+}]_{i}\), were occasionally observed. This activity underlies action potential-induced constrictions (i.e., vasomotion) and could be increased by super-

**Fig. 3. Effects of CPA on agonist-enhanced STD activity.** A–C: STD activity was recorded from smooth muscle in endothelium-lysed segments (left) in the presence of U-46619 (0.1 \(\mu M\)), norepinephrine (50 nM), and thimerosal (1 \(\mu M\)). STD activity was markedly reduced in all cases upon application of 10 \(\mu M\) CPA (right). Scale bars in C apply to all traces. D: STD frequency and amplitude expressed as percentage of control values (i.e., before application of agonist) for U-46619 (0.1 \(\mu M\); \(n = 6\)), norepinephrine (10–50 nM; \(n = 4\)), and thimerosal (1 \(\mu M\); \(n = 5\)) before and in the presence of 10 \(\mu M\) CPA. *\(p < 0.05\) vs. control STD activity before agonist addition; #\(p < 0.05\) vs. STD activity in the presence of agonist.

**Fig. 4. Effects of CPA on U-46619-induced actions on membrane potential and intracellular Ca\(^{2+}\) concentration (\([Ca^{2+}]_{i}\)) in lymphatic smooth muscle with nonfunctional endothelium.** Time scale bar applies to both traces. A: U-46619 caused an increase in STD activity and a transient depolarization and caused near-rhythmic generation of action potentials in a vessel segment exposed to l-NNa (100 \(\mu M\)). B: U-46619 increased \([Ca^{2+}]_{i}\), and \(Ca^{2+}\) transients in an endothelium-denuded vessel segment. Action potentials, STDs, and \(Ca^{2+}\) transients were inhibited by CPA (A and B).

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fusing the preparation with PSS that contained U-46619 (57). This is exemplified in Fig. 4B, where U-46619 (0.1 μM) increased \([\text{Ca}^{2+}]_i\) and induced oscillations that led to more-synchronized spikelike \([\text{Ca}^{2+}]_i\) transients. The frequency of these spikelike transients was in some cases increased with the application of 10 μM CPA, which then blocked this activity while the \([\text{Ca}^{2+}]_i\) was increased to 115 ± 1% of its value in the presence of U-46619 (n = 3; Fig. 4B).

These effects closely compare with the action of CPA on the smooth muscle membrane potential shown in Fig. 4A. In this preparation, U-46619 was added at a concentration that increased STD activity and caused a transient depolarization of the membrane potential and near-rhythmic generation of action potentials. Application of CPA (10 μM) in the continued presence of U-46619 caused a gradual depolarization during which there was an initial increase in the frequency of action potentials and subsequent inhibition of these events (Fig. 4A).

**Direct action of CPA on endothelium.** The effects of CPA on the endothelium were investigated by measuring changes in both \([\text{Ca}^{2+}]_i\) and membrane potential. CPA (10 μM) caused \([\text{Ca}^{2+}]_i\) to increase to 123 ± 2% of control (n = 3), which is a value similar to that obtained in the same preparations with 10 μM ACh (122 ± 5%). However, the kinetics of the onset of the increase in \([\text{Ca}^{2+}]_i\) occurred some 10-fold slower for CPA than for ACh with the times for the response to increase to 50% of peak amplitude 103 ± 7 and 10 ± 1 s, respectively (Fig. 5A).

Endothelial cells of guinea pig mesenteric lymphatic vessels have been shown to be very polarized with a resting membrane potential of about −72 mV (56). In the present study, the mean resting membrane potential was −73.5 ± 1.1 mV (n = 6). This value was depolarized by 1.0 ± 0.4 mV in the first minute of application of 10 μM CPA (n = 6); during this interval, the bath solution was fully exchanged (see METHODS) and in the same cells, ACh induced a marked depolarization (18.2 ± 3.4 mV). However, during longer applications of CPA, a slow depolarization developed (time to peak, 6–7 min) that reached a maximum value of 18.5 ± 3.3 mV, which is close to the maximum response obtained with 10 μM ACh (Fig. 5B). Although the ACh response was sometimes preceded by a small hyperpolarization (1.2 ± 0.7 mV; n = 6; see also Ref. 56), this was not observed in response to CPA.

**DISCUSSION**

The present study investigated the effects of CPA, a selective inhibitor of \([\text{Ca}^{2+}]_i\)-ATPase in smooth muscle SR (6, 8, 26, 45), on pacemaker activity in guinea pig mesenteric lymphatic vessels. CPA caused a complex response that was characterized by changes in the smooth muscle resting membrane potential and the activity of STDs and a slowing in the frequency of \([\text{Ca}^{2+}]_i\) transients. The major action was mediated by direct action of CPA on smooth muscle, although the endothelium also played a role through CPA-induced release of EDNO.

CPA caused the lymphatic smooth muscle membrane potential to hyperpolarize and the STD frequency and amplitude to decrease. The hyperpolarization was shown to be primarily mediated by the CPA-induced production and release of NO from the lymphatic endothelium, as it was strongly reduced in the presence of l-NNA and ODQ as well as in vessels with nonfunctional endothelium. Inhibition of CPA-induced hyperpolarization by glibenclamide further suggests an involvement of EDNO, as NO has been shown to activate ATP-sensitive \(K^+\) channels in the same lymphatic vessels (53). Although NO itself has been shown to reduce STD activity (57), we demonstrate here that the CPA-induced decrease in STD activity was largely independent of EDNO, as CPA still markedly reduced STD activity while endothelial function or NO production was impaired. Consistent with results obtained from other smooth muscles (15, 16, 50), our findings suggest that CPA acts

![Fig. 5. Effects of CPA on \([\text{Ca}^{2+}]_i\), and membrane potential in lymphatic endothelium. A: relative changes in \([\text{Ca}^{2+}]_i\), measured in fura-2-loaded lymphatic endothelium in response to 10 μM CPA (top) and 10 μM ACh (bottom) applied for the duration indicated by horizontal bars. B: intracellular microelectrode recordings demonstrated depolarization of the lymphatic endothelium in response to 10 μM CPA (top) and 10 μM ACh (bottom). Traces in A were obtained from the same vessel segment; traces in B were obtained from a different vessel segment with both recordings made for the same impalement.](http://ajpheart.physiology.org/DownloadedFrom/10.1152/ajpheart.00313.2003)}
directly on lymphatic smooth muscle to inhibit STD generation. At the concentration range used in the present study (1–10 μM), CPA has been reported to selectively alter SR Ca\(^{2+}\)-ATPase function in smooth muscle (see Refs. 6, 45). The decrease in lymphatic smooth muscle STD activity is thus likely to result from the action of CPA on Ca\(^{2+}\)-ATPase, and it correlates with the expected effect of CPA on intracellular Ca\(^{2+}\) handling. Moreover, the small depolarization and initial increase in STD activity that were observed early during CPA superfusion may be explained by the transient increase in [Ca\(^{2+}\)], which is thought to occur as a first event before store depletion, when the reuptake of Ca\(^{2+}\) into the SR is altered (21, 41, 58). Consistent with this, we found that CPA increased [Ca\(^{2+}\)] (see also Ref. 60). The actions of CPA on pacemaker events were also assessed in the presence of norepinephrine and the thromboxane A\(_2\) mimetic U-46619. These agonists have been shown to increase lymphatic pacemaker activity (12, 20, 30, 39, 48, 57) most likely through increased production of IP\(_3\) (9, 35). Pacemaker events (i.e., large STDs or summations 20, 30, 39, 48, 57) most likely through increased production of IP\(_3\) have been shown to increase lymphatic pacemaker activity (12, 41, 58). Consistent with this, we found that CPA increased [Ca\(^{2+}\)], which is associated with the production and release of EDNO, and/or ryanodine receptors (dependent on the tissue; Refs. 1, 4, 5, 18, 38, 43). Importantly, CPA actions to reduce Ca\(^{2+}\)-release events measured by recording STDs are also consistent with this model, as stores would be depleted by CPA-induced block of the store Ca\(^{2+}\)-ATPase (21, 41). However, the finding that there was residual STD activity in the absence or presence of the agonists indicates that CPA-induced inhibition of store function is incomplete for the range of CPA concentrations tested or that additional sources of Ca\(^{2+}\) are available for STDs to occur.

Investigations of CPA action on the lymphatic endothelium revealed an increase in endothelial [Ca\(^{2+}\)], upon CPA stimulation. Although observed here for the first time in lymphatic endothelium, CPA-induced increases in endothelial [Ca\(^{2+}\)] are well documented in many vascular preparations (17, 24, 25). The increase in endothelial [Ca\(^{2+}\)], caused by CPA, like that caused by many other agonists, has been shown to be necessary to the release of endothelium-derived substances such as EDNO (19, 33, 61). The lymphatic endothelium plays an important role in modulating lymphatic pumping (10, 37, 59). In particular, the lymphatic endothelium was shown to slow lymphatic pumping by releasing EDNO both in vitro (32, 40, 54, 59) and in vivo (42). An increase in endothelial [Ca\(^{2+}\)], which is associated with the production and release of EDNO, has also been demonstrated in lymphatic vessels of the guinea pig mesentery in response to ACh (54). This ACh-induced action is accompanied by marked depolarization of the lymphatic endothelium (56). These findings are consistent with the present observations that CPA increases lymphatic endothelial [Ca\(^{2+}\)], and depolarizes membrane potential. However, the rates for both of these changes were much slower than those observed in response to ACh, which is consistent with CPA acting through recruitment of different mechanisms. A gradual increase in [Ca\(^{2+}\)], with a similar time course has been reported for the CPA-induced response of rabbit valvular endothelial cells (25). Although membrane potential was not measured in the latter study, a CPA-induced increase in [Ca\(^{2+}\)], in rabbit aortic valvular endothelial cells has been associated with hyperpolarization of endothelium (36). Hyperpolarizations to vasoactive agonists have been described for many endothelial cells and are proposed to provide the electrochemical driving force for Ca\(^{2+}\) entry into endothelial cells (see Ref. 23) thus promoting the Ca\(^{2+}\)-dependent production of EDNO (27). The finding that the lymphatic endothelium underwent depolarization in response to CPA and ACh does not fit this logic, but such responses have also been reported for some vascular endothelial cells in response to vasoactive substances or Ca\(^{2+}\)-ATPase inhibitors (29, 31, 47).

In conclusion, responses of lymphatic smooth muscle to CPA are suggested to be mediated by two mechanisms: a direct action of CPA through depletion of smooth muscle Ca\(^{2+}\) stores, which inhibits the activity of pacemaker-associated Ca\(^{2+}\) release events measured as STDs, and an action of CPA on endothelium to release EDNO, which causes smooth muscle to hyperpolarize and decrease STD activity. Both actions are proposed to contribute to a decrease in lymphatic pacemaking and the resultant propulsion of lymph.

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REFERENCES
5. Bootman MD, Taylor CW, and Berridge MJ. The thiol reagent, thimerosal, which at the concentration used (1 μM) is known to selectively stimulate Ca\(^{2+}\) release by sensitizing IP\(_3\) and/or ryanodine receptors (dependent on the tissue; Refs. 1, 4, 5, 18, 38, 43). Importantly, CPA actions to reduce Ca\(^{2+}\)-release events measured by recording STDs are also consistent with this model, as stores would be depleted by CPA-induced block of the store Ca\(^{2+}\)-ATPase (21, 41). However, the finding that there was residual STD activity in the absence or presence of the agonists indicates that CPA-induced inhibition of store function is incomplete for the range of CPA concentrations tested or that additional sources of Ca\(^{2+}\) are available for STDs to occur.

Investigations of CPA action on the lymphatic endothelium revealed an increase in endothelial [Ca\(^{2+}\)], upon CPA stimulation. Although observed here for the first time in lymphatic endothelium, CPA-induced increases in endothelial [Ca\(^{2+}\)] are well documented in many vascular preparations (17, 24, 25). The increase in endothelial [Ca\(^{2+}\)], caused by CPA, like that caused by many other agonists, has been shown to be necessary to the release of endothelium-derived substances such as EDNO (19, 33, 61). The lymphatic endothelium plays an important role in modulating lymphatic pumping (10, 37, 59). In particular, the lymphatic endothelium was shown to slow lymphatic pumping by releasing EDNO both in vitro (32, 40, 54, 59) and in vivo (42). An increase in endothelial [Ca\(^{2+}\)], which is associated with the production and release of EDNO, has also been demonstrated in lymphatic vessels of the guinea pig mesentery in response to ACh (54). This ACh-induced


