VEGF and ATP act by different mechanisms to increase microvascular permeability and endothelial \([\text{Ca}^{2+}]_i\)

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VEGF and ATP act by different mechanisms to increase microvascular permeability and endothelial \([\text{Ca}^{2+}]_i\). Am J Physiol Heart Circ Physiol 279: H1625–H1634, 2000.—Vascular endothelial growth factor (VEGF) increases hydraulic conductivity \((L_p)\) by stimulating \([\text{Ca}^{2+}]_i\) influx into endothelial cells. To determine whether VEGF-mediated \([\text{Ca}^{2+}]_i\) influx is stimulated by release of \([\text{Ca}^{2+}]_i\) from intracellular stores, we measured the effect of \([\text{Ca}^{2+}]_i\) store depletion on VEGF-mediated increased \(L_p\) and endothelial intracellular \([\text{Ca}^{2+}]_i\) concentration \([\langle \text{Ca}^{2+}\rangle_i]\) of frog mesenteric microvessels. Inhibition of \([\text{Ca}^{2+}]_i\) influx by perfusion with \(\text{NiCl}_2\) significantly attenuated VEGF-mediated increased \([\langle \text{Ca}^{2+}\rangle_i]\), and \([\text{Ca}^{2+}]_i\) depleted and under conditions that prevented ATP-mediated increases in \([\langle \text{Ca}^{2+}\rangle_i]\), or the increase in \(L_p\). In contrast, ATP-mediated increases in both \([\langle \text{Ca}^{2+}\rangle_i]\), and \(L_p\) were inhibited by thapsigargin perfusion, demonstrating that ATP stimulated store-mediated \([\text{Ca}^{2+}]_i\) influx. VEGF also increased Mn\(^{2+}\) influx after perfusion with thapsigargin, whereas ATP did not. These data showed that VEGF increased \([\langle \text{Ca}^{2+}\rangle_i]\), and \(L_p\), even when \([\langle \text{Ca}^{2+}\rangle_i]\) stores were depleted and under conditions that prevented ATP-mediated increases in \([\langle \text{Ca}^{2+}\rangle_i]\), and \(L_p\). This suggests that VEGF acts through a \([\text{Ca}^{2+}]_i\) store-independent mechanism, whereas ATP acts through \([\text{Ca}^{2+}]_i\) store-mediated \([\text{Ca}^{2+}]_i\) influx.

vascular endothelial growth factor; vascular permeability; endothelial calcium; calcium stores; intracellular calcium concentration; adenosine 5’-triphosphate
that stimulation of receptor tyrosine kinases results in PLC-γ activation, which produces IP₃. This acts on IP₃ receptors on the endoplasmic reticulum to release Ca²⁺ from internal stores (5). This may be great enough to result in a transient increase in [Ca²⁺]ᵢ. A second possibility is that release of Ca²⁺ from internal stores generates a Ca²⁺ release-activated Ca²⁺ (CRAC) influx across the plasma membrane, by capacitative Ca²⁺ entry (12), and it is the subsequent influx of Ca²⁺ that results in increased [Ca²⁺]ᵢ. This pathway has been shown to result in Ca²⁺ influx into endothelial cells in culture when stimulated with ATP, but it is not currently known whether this pathway is the mechanism by which [Ca²⁺]ᵢ increases in endothelial cells in vivo or whether it is responsible for the increases in permeability that result from exposure to ATP or VEGF. It has not even been shown that VEGF does cause Ca²⁺ release from intracellular stores in endothelial cells in vivo or whether the increase in permeability is a result of this Ca²⁺ release. A third hypothesis is that VEGF acts to increase Ca²⁺ influx across the plasma membrane through a store-independent mechanism, possibly by acting on receptor-operated Ca²⁺ channels. To determine which, if any, of these three hypotheses is correct, we measured the increase in [Ca²⁺]ᵢ, and permeability brought about by VEGF and ATP under conditions where the Ca²⁺ stores have been depleted and therefore do not release Ca²⁺. Ca²⁺ stores may be depleted by perfusion of vessels with thapsigargin, an irreversible inhibitor of the endoplasmic reticulum Ca²⁺-ATPase (26). The sarcoendoplasmic reticulum Ca²⁺-ATPase (SERCA) pumps Ca²⁺ from the cytoplasm into the endoplasmic reticulum. There is a slow but steady leak of Ca²⁺ from the stores into the cytoplasm, so inhibition of this protein by use of thapsigargin results in a steady depletion of Ca²⁺ stores. Although thapsigargin has been extensively used in endothelial (and other) cells in culture, there has been only one study to date investigating the effect of thapsigargin on [Ca²⁺]ᵢ of endothelial cells of the microvasculature in vivo (37). Therefore, we also investigated the effect of thapsigargin on endothelial [Ca²⁺]ᵢ and permeability. Some of these results have previously been published as abstracts (2, 34).

METHODS

Frog preparation. All experiments were carried out on male leopard frogs (20–35 g). Hydraulic conductivity (Lᵥ) measurements were made in Rana temporaria supplied by Blades, and Ca²⁺ measurements were made in Rana pipiens supplied by J. M. Hazen, V.T. All chemicals were purchased from Sigma unless otherwise specified. ATP was perfused at 30 μM and VEGF at 1 nM, because these doses have previously been shown to generate a reproducible increase in both [Ca²⁺]ᵢ and Lᵥ. Thapsigargin (Calbiochem) was perfused at 100 nM, because this has been shown to effectively inhibit SERCA in a variety of animal species and does not result in inhibition of other Ca²⁺ pumps.

Measurement of Lᵥ. Frogs were anesthetized by immersion in 1 mg/ml MS-222 (3-aminobenzoic acid ethyl ester) in water, and anesthesia was maintained by superfusion of the gut with 0.1–0.25 mg/ml MS-222 in frog Ringer solution (111 mM NaCl, 2.4 mM KCl, 1 mM MgSO₄, 1.1 mM CaCl₂, 0.20 mM NaHCO₃, 2.63 mM HEPES acid, and 2.37 mM HEPES sodium salt). The pH of this solution was 7.40 ± 0.02 at room temperature. The animal was laid supine, and the limbs were secured lightly. A small incision (8–10 mm) was made in the right lateral skin and muscular body wall. The distal ileum was floated out and carefully draped over a 1-cm-diameter transparent quartz pillar. The microvessels in the mesentery were visualized under an inverted microscope (Leica DMIL). A video camera (Panasonic WVBP32, 8 mm) was attached to the top of the microscope to allow for binocular visualization and simultaneous recording of a 270-μm segment of the vessel (out of a total length of 800–2,000 μm). The video was connected through an electronic timer (ForA VT33) to a video cassette recorder (Panasonic AG7350; Panasonic, Bracknell, UK). The upper surface of the mesentery was kept continuously superfused with frog Ringer solution during the entire time that it was exposed. All experiments were carried out at room temperature (20–22°C). At the end of the experiment, the frog was killed by destruction of the cranium.

The Lᵥ of perfused mesenteric microvessels was measured by use of the Landis microocclusion method previously described (27), which has been extensively discussed in the literature (10) and adapted to measure rapid changes in Lᵥ (3). Baseline Lᵥ was defined as the conductivity during perfusion with 1% BSA in frog Ringer solution, adjusted to pH 7.4 with 0.115 M NaOH. Microvessels were selected that contained freely flowing blood, had no white cells adhering to or rolling along the wall, were at least 800-μm long with no side branches, and had a baseline Lᵥ of <10 × 10⁻⁷ cm²·s⁻¹·cmH₂O⁻¹. Microvessels chosen for Lᵥ measurement were either true capillaries (divergent flow at one end and convergent at the other) or first-order venules (convergent flow from two true capillaries at one end and convergent flow at the other) and had a diameter of 12–30 μm (we have previously shown that permeability responses to VEGF are not dependent on vessel size, see Ref. 3). Glass micropipettes were manufactured from pulled capillary tubes (outer diameter 1.5 mm, Clark ElectroMed) and beveled to form a sharp tip 10–17 μm in diameter. The vessel was cannulated with a micropipette filled with 1% BSA in frog Ringer solution and rat red blood cells as flow markers. The rat red blood cells were collected by direct cardiac puncture of 5% halothane-anesthetized rats and were washed three times in frog Ringer solution before use. Rats were killed by cervical dislocation while still anesthetized. The micropipette was clamped in a holder (WPI, Stevenage) and connected to a water manometer. The pipette was refilled with solution when required by use of a refilling system based on that described by Neal (29). Lᵥ was measured by occluding the vessel with a glass rod for 3–7 s while perfusing at a pressure of 30 cmH₂O. The vessel was then allowed to flow freely for at least 7 s before another occlusion was made. Refilling was observed as a change in the hematocrit of the perfusate, and the vessel was occluded immediately for 3–5 s as soon as possible to measure Lᵥ. The occlusion was released, and Lᵥ could then be measured approximately every 10 s. Lᵥ measurements were performed every 10–20 s during perfusion with test compounds. All perfusates contained rat red blood cells as flow markers.

Calculation of Lᵥ. The transcapillary water flow per unit area of capillary wall (Jₛ/S) was calculated from the initial velocity of the red blood cells (d/dt, change in length over change in time) after occlusion, the capillary radius (r), and the length between the marker cell and the point of occlusion.
(l), all of which were measured offline from the videotape

\[
(J_v/S) = (dI_v/dt) \cdot [r/(2 \cdot l)]
\]

The \( L_p \) was calculated from the Starling equation

\[
L_p = (J_v/S) / \Delta P
\]

where \( \Delta P \) is the effective hydrostatic and oncotic pressure difference between the capillary and the interstitium. The capillary pressure was set at 30 cmH₂O, so \( \Delta P \) was 26.4 cmH₂O (1% BSA has an effective oncotic pressure of 3.6 cmH₂O), with the assumption that tissue pressure was negligible, and tissue oncotic pressure was equivalent to that in the superfusate (zero).

**Measurement of \([Ca^{2+}]_i\), \([Ca^{2+}]_v\)** was measured in frog mesenteric microvessels as previously described (4). In brief, the frog was pithed and laid supine, and the limbs were secured to a supporting tray. The abdominal cavity was opened by making incisions on both sides of the trunk and across the midline. The body wall flaps was then lifted back over the upper body to expose the viscera, which were held in place by cotton wool soaked in Ringer. The mesentery was floated over a glass coverslip attached to the supporting tray and held in place by pinning of the gut to the edge of the coverslip. This allowed for observation of a perfused microvessel with the short working distance lens necessary for fluorescence measurement. The upper surface of the mesentery was continuously superfused with Ringer, and the temperature of the superfusate was kept at 20–22°C. Vessels chosen for cannulation were postcapillary venules (first-, second-, and third-order venules) of diameter 25–40 μm. Vessels were visualized under an epifluorescence microscope (Leitz Diavert) equipped with quartz optics in the excitation pathway, a photomultiplier tube (Leitz MPV) and excitation filter changer (Kinetic) under computer control, and a 100-W mercury lamp. A selected vessel was cannulated and perfused with 1% BSA in Ringer. Fluorescence intensity \( (I) \), collected by a dry Fluortar lens \((20 \times, \text{numerical aperture } 0.75)\), was measured from a window 150-μm long and 40-μm wide that was placed ~200 μm downstream of the cannulation site. \( I \) values at excitation wavelengths of 340 ± 5 and 380 ± 5 nm (selected by 2 narrow band interference filters) and emission at 500 ± 55 nm were collected by use of a 0.25-s exposure to give an initial background \( \bar{I} \), for each vessel that could be used to estimate fura loading. All \( I \) values were measured during perfusion at a pressure of 30 cmH₂O. The vessel was then perfused with frog Ringer containing 5 μM fura 2-AM and 1% BSA for 60–120 min in the dark. The vessel was briefly examined during this time by illumination with 380-nm light to check that even loading was occurring. Once the \( \bar{I} \) had reached 6–10× the background, the vessel was perfused for 10 min with 1% BSA to give a baseline \( Ca^{2+} \) reading.

After the baseline \( \bar{I} \) was measured, the vessel was perfused with various pharmacological agents (see RESULTS) in Ringer containing 1% BSA. Finally, the vessel was perfused with 1% BSA containing 5 mM MnCl₂ and nominally 0 μM \( Ca^{2+} \). This final perfusion quenched the fluorescence, presumably from the \( Ca^{2+} \)-sensitive form of fura 2, and a second background intensity reading was taken. However, we noticed that the rate of quench appeared to be much slower after perfusion with thapsigargin. We therefore measured the rate of quenching after perfusion with Mn \( \text{Cl}_2 \), by measurement of \( \bar{I} \), at 360-nm excitation \((I_{3606}; \text{see DISCUSSION})\). After the final \( I \) measurement was made, the animal was killed by decapitation. Vessels were accepted that had a diffuse fluorescence throughout the endothelial cells, with more intense fluorescence around the nuclei (due to increased cell thickness around the nucleus), and a \( I \) at least 6× background.

**Calculation of \([Ca^{2+}]_v\), \([Ca^{2+}]_v\)** was calculated from the equation (16, 35)

\[
[Ca^{2+}]_v \equiv K(R - 0.85)(/0.85R_{\text{max}} - R)
\]

where \( R \) is the normalized ratio of \( I_v \) calculated as

\[
R = R_{\text{exp}} / R_{\text{min}}
\]

where \( R_{\text{exp}} = (I_{340} - B_{340})/(I_{380} - B_{380}) \). \( I_{340} \) is the \( I \) with excitation at 340 nm, \( I_{380} \) is the \( I \) with excitation at 380 nm, and \( B_{340} \) and \( B_{380} \) are the background \( I \) values at excitations of 340 and 380 nm, respectively (measured as the \( I \) after Mn \( \text{Cl}_2 \) quenching). \( R_{\text{max}} \) is the in vitro ratio at saturating \( Ca^{2+} \) concentration normalized to \( R_{\text{min}} \). \( R_{\text{min}} \) is the in vitro ratio for zero \( Ca^{2+} \) concentration, and \( K \) is the product of the effective dissociation constant for fura 2 and the ratio of the \( I \) in vitro \( I_{340} \), for zero and saturating \( Ca^{2+} \). \( K \) was estimated from an in vitro calibration curve for fura 2, as previously described (4).

**Data analysis and statistics.** To compare responses of different vessels, individual readings from single vessels were normalized relative to baseline and then time averaged in 15-s (for \( L_p \) measurement) or 10-s (for \([Ca^{2+}]_v\), measurement) bins, starting immediately after perfusion with agonist. The means ± SE of the time-averaged measurements for all the vessels within that group were then calculated. The peak of the time-averaged data was therefore always lower than the actual peak value for the vessel, by definition. The mean peak values for the vessel will therefore be greater than the time-averaged and vessel-averaged \([Ca^{2+}]_v\), and \( L_p \).

Multiple-way comparisons of data were carried out using ANOVA with Bonferroni post hoc tests. Two-way comparisons of data were carried out using paired t-tests. Where data were not normally distributed, a Friedman test was performed to provide significance for repeated measures, with Dunn’s post hoc tests.

**RESULTS**

**Effect of inhibition of \( Ca^{2+} \) influx on VEGF-induced changes in endothelial cell \([Ca^{2+}]_v\).** Four vessels were perfused with 1 nM VEGF, and \([Ca^{2+}]_v\) was measured. To investigate the contribution of \( Ca^{2+} \) influx across the plasma membrane on VEGF-mediated increased \([Ca^{2+}]_v\), we used 5 mM NiCl₂ to inhibit \( Ca^{2+} \) influx into endothelial cells in vivo, as previously described (13). Time-averaged data are shown in Fig. 1 for vessels perfused with and without NiCl₂. In four vessels perfused with 1 nM VEGF, a significant 108 ± 26 nM transient increase in \([Ca^{2+}]_v\), was observed from 79 ± 10 to 187 ± 33 nM (P < 0.01). When 5 mM NiCl₂ was included in the perfusate and superfusate, there was no significant increase in \([Ca^{2+}]_v\). After washout of nickel with normal Ringer, perfusion with 1 nM VEGF again caused a significant 190 ± 24 nM transient rise in \([Ca^{2+}]_v\), from 95 ± 15 to 285 ± 19 nM (P < 0.001). This was significantly (P < 0.001) higher than the increase in \([Ca^{2+}]_v\), in the presence of nickel. The increase in \( Ca^{2+} \) was therefore significantly attenuated by perfusion and superfusion with 5 mM NiCl₂

**Effect of thapsigargin on \([Ca^{2+}]_v\), and \( L_p \).** To investigate the effect of thapsigargin on endothelial \([Ca^{2+}]_v\), 16 vessels were perfused with 100 nM thapsigargin,
and \([\text{Ca}^{2+}]_i\) was measured (see Fig. 2A). Perfusion with thapsigargin resulted in a significant transient increase in \([\text{Ca}^{2+}]_i\) from 96 ± 11 to 278 ± 28 nM \((P < 0.001)\), usually within 5 min and in all cases within 15 min. \([\text{Ca}^{2+}]_i\) then returned to control values until it reached a significantly lower \((P < 0.001)\) sustained concentration of 105 ± 14 nM after an additional 5 min, which was not significantly different from the concentration before thapsigargin (not significant, Fig. 2).

Twelve additional vessels were perfused with thapsigargin, and \(L_p\) was measured. A representative trace is shown in Fig. 2B. Thapsigargin perfusion resulted in a transient increase in \(L_p\) to 12.8 ± 2.8-fold greater than baseline \((33 ± 11 \text{ cm} \cdot \text{s}^{-1} \cdot \text{cmH}_2\text{O}^{-1}, P < 0.01; \text{Fig. 2C, right})\). The \(L_p\) then returned to control values over the following 5–10 min and reached an average of 4.1 ± 1.5 \(\times 10^{-7}\) cm\(\cdot\)s\(^{-1}\)\cdot\text{cmH}_2\text{O}^{-1}, which was not significantly different from the baseline \(L_p\) \((P > 0.05)\).

**Effect of thapsigargin on the increase in \(L_p\) brought about by VEGF.** Measurements of \(L_p\) were made on nine vessels perfused with 1 nM VEGF (Table 1). Perfusion with VEGF caused an immediate and transient 5.4 ± 1.1-fold increase in \(L_p\), as previously described \((P < 0.05, \text{paired } t\text{-test})\). VEGF was then washed out for 20 min to prevent further responses being masked by the tachyphylaxis previously described. After 20-min perfusion with thapsigargin, \(L_p\) was not significantly different from control. Subsequent perfusion with 1 nM VEGF in the presence of thapsigargin caused a rapid 7.5 ± 2.7-fold increase in \(L_p\), which was on average not significantly different from the response to VEGF before thapsigargin perfusion \((P > 0.05)\). The time-averaged responses to VEGF in the presence and absence of thapsigargin are shown in Fig. 3A.
before ATP). After 20-min perfusion with thapsigargin, SE.
perfusion immediately before agonist perfusion. Values are means during perfusion with 30 nM VEGF perfusion before (L pretreatment with 1 nM TG. The increase in ATP, with and without pretreatment with thapsigargin. Perfusion of vessels with ATP before thapsigargin stimulated a rapid 5.46-fold increase in Lp, from a mean baseline of 3.81.4 cm s⁻¹ cmH₂O⁻¹ to a peak of 19 4.9 × 10⁻⁷ cm s⁻¹ cmH₂O⁻¹. The Lp then returned toward control values (7.9 ± 3.1 × 10⁻⁷ cm s⁻¹ cmH₂O⁻¹, P > 0.05 compared with baseline before ATP). After 20-min perfusion with thapsigargin, ATP perfusion did not increase Lp (1.1 ± 0.1-fold, P < 0.001 vs. the increase before thapsigargin), from a base of 8.1 ± 3.0 (P > 0.05 vs. before thapsigargin) to a peak of 8.9 ± 3.2 × 10⁻⁷ cm s⁻¹ cmH₂O⁻¹ (P > 0.05 vs. base). Some representative traces of Lp data are shown in Fig. 4. Figure 4A shows that Lp increases in response to VEGF and without thapsigargin perfusion. Figure 4B shows a vessel in which Lp was measured during perfusion with ATP; it was then perfused with thapsigargin for 20 min and then with ATP, and Lp was measured, followed by washout with thapsigargin and BSA; and Lp was then measured during perfusion of the same vessel with VEGF. In this and two other vessels similarly perfused, no increase in Lp was seen during perfusion with ATP, whereas subsequent perfusion of the same vessel with VEGF resulted in a significant transient increase in Lp. Furthermore, the effect of a different SERCA inhibitor, cyclopiazonic acid (CPA), on the VEGF- and ATP-mediated increased permeability was also examined in one vessel (see Fig. 4C). ATP did not increase Lp in the presence of CPA, whereas VEGF resulted in a significant transient increase in Lp in that same vessel. CPA therefore had the same effect as thapsigargin. Finally, to ensure that Ca²⁺ stores had been depleted, we perfused one vessel with thapsigargin and 1 µM ionomycin to stimulate release from stores. Once the Lp had returned toward control values, we then perfused that vessel with VEGF. VEGF still gave a typical transient increase in permeability (Fig. 4D).

**Effect of thapsigargin on the increase in [Ca²⁺]i brought about by VEGF.** Measurements of [Ca²⁺]i were made in nine vessels perfused with 1 nM VEGF (Table 1). Perfusion with VEGF stimulated a 111 ± 24 nM rapid, transient rise in [Ca²⁺]i from 71 ± 12 to 182 ± 20 nM (P < 0.001). After vessels were perfused with thapsigargin, perfusion with 1 nM VEGF and thapsigargin produced a significant rapid, transient 211 ± 44 nM increase in [Ca²⁺]i, from 85 ± 20 to 296 ± 90 nM (P < 0.001), which was, on average, not significantly different from that before thapsigargin (P > 0.05). The time-averaged increase in Ca²⁺ across all nine vessels is shown in Fig. 5A (time-averaged data will not show the full extent of the increase in Ca²⁺ because each measurement in each vessel is the average of 4 Ca²⁺ measurements, only 1 of which could have been the peak response). It can be seen that there was no significant difference in the VEGF response whether thapsigargin was present or absent.

### Table 1. Effect of TG pretreatment on ATP- and VEGF-mediated increases in [Ca²⁺]i and Lp

<table>
<thead>
<tr>
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<th>Lp × 10⁻⁷ cm s⁻¹ cmH₂O⁻¹</th>
<th>[Ca²⁺]i nM</th>
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<tr>
<td></td>
<td>Before TG</td>
<td>With TG</td>
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<tr>
<td>VEGF</td>
<td>Base</td>
<td>Peak</td>
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<tr>
<td></td>
<td>2.6 ± 0.6</td>
<td>13.2 ± 4.3*</td>
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<tr>
<td>ATP</td>
<td>1.4 ± 1.4</td>
<td>8.1 ± 3.0</td>
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Values are means ± SE for hydraulic conductivity (Lp) and intracellular calcium concentration ([Ca²⁺]i) before and during vascular endothelial growth factor (VEGF; n = 9 vessels) or ATP (n = 7 vessels) perfusion with and without thapsigargin (TG) pretreatment. *Significantly greater than baseline (P < 0.05).

These results are in marked contrast to those obtained with ATP. Figure 3B shows time-averaged data of Lp measurements made in seven vessels perfused with ATP, with and without pretreatment with thapsigargin. Perfusion of vessels with ATP before thapsigargin stimulated a rapid 5.4 ± 0.6-fold increase in Lp, from a mean baseline of 3.8 ± 1.4 to a peak of 19 ± 4.9 × 10⁻⁷ cm s⁻¹ cmH₂O⁻¹. The Lp then returned toward control values (7.9 ± 3.1 × 10⁻⁷ cm s⁻¹ cmH₂O⁻¹, P > 0.05 compared with baseline before ATP). After 20-min perfusion with thapsigargin,
In contrast, 100 nM thapsigargin perfusion did affect the ATP-mediated increases in [Ca\(^{2+}\)]\(i\). Measurements of [Ca\(^{2+}\)]\(i\) were made in seven vessels perfused with 30 μM ATP. Time-averaged data are shown in Fig. 5B. In these vessels, ATP caused a rapid 195 ± 22 nM rise in [Ca\(^{2+}\)]\(i\), from a baseline of 93 ± 18 to a peak of 288 ± 34 nM (P < 0.001). After vessels were perfused with thapsigargin for 20 min, perfusion with 30 μM ATP did not significantly increase [Ca\(^{2+}\)]\(i\) (P > 0.05). The baseline [Ca\(^{2+}\)]\(i\) after thapsigargin perfusion (129 ± 20 nM) was not significantly different from the baseline before thapsigargin (but after ATP, 105 ± 12 nM, P > 0.05), and the peak [Ca\(^{2+}\)]\(i\) reached during ATP and thapsigargin perfusion (192 ± 28 nM) was significantly (P < 0.01) smaller than the peak Ca\(^{2+}\) reached with ATP alone (288 ± 34 nM; see Table 1) and not significantly different from the baseline (P > 0.05). Perfusion of the vessel with thapsigargin significantly attenuated the ATP-mediated [Ca\(^{2+}\)]\(i\) increase, suggesting that perfusion with 100 nM thapsigargin resulted in Ca\(^{2+}\) store depletion.

In three vessels, both VEGF and ATP were given with thapsigargin sequentially. These traces are shown in Fig. 6. Figure 6A shows that in a single vessel, VEGF increases [Ca\(^{2+}\)]\(i\) with thapsigargin at least as effectively as it did before thapsigargin. ATP did not increase [Ca\(^{2+}\)]\(i\), in the presence of thapsigargin, but VEGF was still able to increase [Ca\(^{2+}\)]\(i\) in the continued presence of thapsigargin, even after exposure to ATP. Figure 6B shows again that both VEGF and ATP increase [Ca\(^{2+}\)]\(i\) before thapsigargin, but ATP repeatedly fails to increase [Ca\(^{2+}\)]\(i\), in the presence of thapsigargin, and VEGF consistently and repeatedly increases [Ca\(^{2+}\)]\(i\) in a similar manner to that before thapsigargin perfusion. This shows that VEGF can stimulate a second increase in [Ca\(^{2+}\)]\(i\), even during continuous thapsigargin perfusion. Figure 6C shows that another SERCA inhibitor, CPA, is also capable of inhibiting the effect of ATP but does not affect VEGF-stimulated [Ca\(^{2+}\)]\(i\).

VEGF stimulates Mn\(^{2+}\) influx in the presence of thapsigargin. As part of the methodology for the measurement of [Ca\(^{2+}\)]\(i\), in endothelial cells in vivo by use of fluorescent indicator dyes such as fura 2, it was necessary to determine the background \(I_f\) by quenching the Ca\(^{2+}\)-sensitive fura 2. To do this, at the end of the experiment, the vessels were perfused with Mn\(^{2+}\), which enters cells through Ca\(^{2+}\) channels and quenches the Ca\(^{2+}\)-sensitive indicator. The rate of quench of the indicator is a measure of the rate of Mn\(^{2+}\) influx (and, by inference, Ca\(^{2+}\) influx across the plasma membrane). We noticed that the rate of quench with thapsigargin appeared to be slower than usual. We therefore investigated the change in the rate of quench stimulated by VEGF and ATP to determine whether VEGF or ATP could indeed stimulate quenching of fura 2. At the end of the experiments to measure Ca\(^{2+}\) changes, the vessels were perfused with frog Ringer, which was nominally Ca\(^{2+}\) free and contained 5 mM Mn\(^{2+}\) and 1% BSA. The \(I_f\) at 360 nm (the isosbestic point for fura 2, the wavelength at which there is no Ca\(^{2+}\) sensitivity) was then measured for 10–20 s. Four vessels were perfused with 1% BSA with Mn\(^{2+}\) and then 1 nM VEGF with 1% BSA and Mn\(^{2+}\). In another four vessels, vessels were perfused with the 1% BSA and Mn\(^{2+}\); then with 1% BSA, Mn\(^{2+}\), and 30 μM ATP; and then with 1% BSA, Mn\(^{2+}\), and 1 nM VEGF. \(I_f\) at 360 nm was measured for 10–20 s after each cannulation.

The rate of quench of a single vessel is expressed in Fig. 7 as the \(I_f\) as a proportion of the initial intensity.
(\text{I}_0). The slope of the curve is proportional to the rate of Mn\textsuperscript{2+} entry. It can be seen from Fig. 7 that perfusion of a vessel with 30 μM ATP did not significantly increase the rate of quench of the dye, i.e., perfusion with 30 μM ATP in the presence of thapsigargin did not result in increased Ca\textsuperscript{2+} influx. However, perfusion with VEGF did increase the rate of quench and resulted in a rapid, complete quench. VEGF was therefore capable of stimulating Ca\textsuperscript{2+} influx in the presence of thapsigargin, whereas ATP could not. Quench rates were measured for 1% BSA and VEGF in four vessels and for BSA, ATP, and VEGF in four vessels. The quench rates for BSA and ATP were not different from each other (1.0 ± 0.2%/s for BSA (n = 8) and 1.3 ± 0.7%/s for ATP (n = 4)) but were significantly higher during VEGF perfusion [7.6 ± 5.3%/s (n = 8), P < 0.01 compared with both ATP and BSA].

DISCUSSION

The ability to increase [Ca\textsuperscript{2+}]\textsubscript{i} was one of the first effects of VEGF described during its initial purification.
Ca\(^{2+}\) influx rather than store release. This hypothesis is also consistent with previous data showing that the increase in permeability brought about by VEGF can also be inhibited by perfusion with nickel (4). However, thapsigargin- and CPA-insensitive Ca\(^{2+}\) stores have been described in endothelial cells in vitro. It has been shown that [Ca\(^{2+}\)]\(_i\) may increase by release of Ca\(^{2+}\) from mitochondrial Ca\(^{2+}\) stores (23). If VEGF stimulated significant Ca\(^{2+}\) release from mitochondria, inhibition of Ca\(^{2+}\) influx might not be expected to affect the increase in [Ca\(^{2+}\)]\(_i\), so dramatically. We cannot rule out a contribution from mitochondria store, however, even though inhibition of Ca\(^{2+}\) influx with nickel greatly reduces the magnitude of [Ca\(^{2+}\)]\(_i\) increase after VEGF.

The rate of entry of Ca\(^{2+}\) can be estimated by use of the quenching properties of Mn\(^{2+}\) on Ca\(^{2+}\) indicator dyes. Jacob (21) has previously shown that agonists that stimulate Ca\(^{2+}\) influx in endothelial cells in culture result in increased rate of quench of fura 2. The experiments described here were not specifically designed to measure Ca\(^{2+}\) influx (we do not have data on the rate of quench of vessels not perfused with thapsigargin, for instance) and cannot be used to determine general mechanisms regulating Ca\(^{2+}\) influx. However, the observation that VEGF stimulates the rate of quenching of fura 2 by Mn\(^{2+}\) in the presence of thapsigargin but ATP did not increase Mn\(^{2+}\) influx, strongly suggests that VEGF acts to increase Ca\(^{2+}\) influx through a pathway which is different from that stimulated by ATP (22).

**VEGF and ATP act through different mechanisms.** ATP is known to stimulate P\(_{2Y}\) purinoreceptors, which results in IP\(_3\) production and release of Ca\(^{2+}\) from endoplasmic reticulum (33). It is this release of Ca\(^{2+}\) from intracellular stores that stimulates Ca\(^{2+}\) entry through a passive conductance pathway, possibly by hyperpolarization of the endothelial cell by stimulation of Ca\(^{2+}\)-activated potassium channels (K\(_{Ca}\)) (9). Our data suggest that the Ca\(^{2+}\) influx resulting from stimulation of endothelial cells with ATP in vivo is brought about by release of Ca\(^{2+}\) from intracellular stores, in the same manner as it is in vitro. It has previously been shown that the permeability increase caused by ATP is also dependent on activation of Ca\(^{2+}\) influx. The increase in permeability is dependent on extracellular Ca\(^{2+}\) and can be inhibited by reducing the driving force for Ca\(^{2+}\) influx (18). It may be hypothesized that inhibition of the stimulus for Ca\(^{2+}\) influx, the release of Ca\(^{2+}\) from intracellular stores, would inhibit the permeability increase. Figure 3B shows that perfusion with thapsigargin for 20 min successfully inhibited the permeability increase brought about by ATP. This shows that the conditions described here effectively blocked store-dependent, Ca\(^{2+}\) influx-mediated permeability increases. They serve as useful controls to differentiate between store-dependent and -independent increases in permeability.

The VEGF-mediated increase in [Ca\(^{2+}\)]\(_i\) and \(L_p\) is consistent with the hypothesis that VEGF acts through a store-independent mechanism. Stimulation of Ca\(^{2+}\) influx independently of release from internal stores
has been shown to occur through at least three classes of plasma membrane Ca^{2+} channels. These include voltage-operated Ca^{2+} channels, which are not present in endothelial cells either in culture or in vivo (9, 31); receptor-operated Ca^{2+} channels, which do not include any of the known VEGF receptors (30); and second messenger-operated, store-independent Ca^{2+} channels (6). The discovery of store-independent Ca^{2+} channels is currently proceeding apace with the cloning and characterization of novel TrpC channels (32). TrpC3 and TrpC6 Ca^{2+} channels have recently been described as being activated by second messengers such as diacylglycerol (19), which is known to be produced by VEGF stimulation, but it is not known whether these channels are activated by VEGF. They have been shown to be activated by other growth factors acting on tyrosine kinase receptors, however (25). The identification of the channels through which VEGF stimulates Ca^{2+} influx may provide a target for drug design to target conditions associated with VEGF over- or underproduction, including all tumors, diabetic retinopathy, and heart disease.

**Effect of thapsigargin on [Ca^{2+}]i and vascular permeability.** Thapsigargin has been extensively used in many studies of endothelial [Ca^{2+}]i regulation. However, all of the studies where endothelial [Ca^{2+}]i has been measured during exposure to thapsigargin have been carried out in cultured endothelial cells (usually from large arteries or veins), with the exception of one study, which measured [Ca^{2+}]i in endothelial cells of the lung microvasculature in situ (37). This study unfortunately did not measure Ca^{2+} changes during thapsigargin perfusion but showed that Ca^{2+} waves were inhibited by thapsigargin perfusion. The data presented here are therefore the first description of the effects of SERCA inhibitors on [Ca^{2+}]i in intact endothelial cells of vessels in vivo. Figure 2 shows that thapsigargin perfusion into microvessels causes a transient increase in [Ca^{2+}]i. This is similar in some respects to the effect of thapsigargin on endothelial cells in vitro (11) but also differs in one important aspect. Application of thapsigargin to endothelial cells in vitro results in a transient increase in [Ca^{2+}]i, which peaks at approximately the same level as described here (400 ± 110 nM) and does not return to the control values but maintains a high sustained level (11). This sustained increase is 70% of the peak increase. This is significantly greater than the sustained level measured in this study, which is only 8 ± 10% of the peak increase (P < 0.05). This finding implies that Ca^{2+} store depletion does not result in a sustained [Ca^{2+}]i increase in endothelial cells in vivo. Possible differences between in vivo and in vitro settings include the extent to which Ca^{2+} extrusion is activated and the contribution of K_{Ca} to the regulation of membrane hyperpolarization and hence part of the driving force for Ca^{2+} entry (14). The finding that store depletion does not result in a sustained increase in [Ca^{2+}]i is somewhat contradictory to our expectation, and this question deserves further attention. However, the mechanisms linking the thapsigargin-induced Ca^{2+} release to the time course of change in [Ca^{2+}]i and L_{p} are not the focus of this investigation.

The experiments described in this paper show that the VEGF-mediated increase in [Ca^{2+}]i in endothelial cells in vivo can be attenuated by inhibition of Ca^{2+} influx. The increase in [Ca^{2+}]i and the associated increase in L_{p} occur even after Ca^{2+} store depletion. This is in direct contrast to the increases in permeability and [Ca^{2+}]i brought about by ATP, which were inhibited by store depletion. In addition, VEGF but not ATP stimulated Mn^{2+} entry into endothelial cells after store depletion. VEGF therefore acts through a different signaling pathway from ATP, and this evidence suggests that VEGF acts to increase [Ca^{2+}]i, Ca^{2+} influx and microvascular permeability through a Ca^{2+}-store-independent mechanism.

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