Evidence of a role for TRPC channels in VEGF-mediated increased vascular permeability in vivo

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Pocock, T. M., R. R. Foster, and D. O. Bates. Evidence of a role for TRPC channels in VEGF-mediated increased vascular permeability in vivo. Am J Physiol Heart Circ Physiol 286: H1015–H1026, 2004. First published October 9, 2003; 10.1152/ajpheart.00826.2003.—Vascular endothelial growth factor (VEGF) increases vascular permeability by stimulating endothelial Ca²⁺ influx. Here we provide evidence that links VEGF-mediated increased permeability and endothelial intracellular Ca²⁺ concentration ([Ca²⁺]i) with diacylglycerol (DAG)-mediated activation of the transient receptor potential channels (TRPCs). We used the Landis-Michel technique to measure changes in hydraulic conductivity (Lp) and fluorescence photometry to quantify changes in endothelial [Ca²⁺]i, in individually perfused Rana mesenteric microvessels in vivo and transfected nonendothelial cells in vitro. The membrane-permeant DAG analog 1-oleoyl-2-acetyl-sn-glycerol (OAG, 100 μM), which is known to increase Ca²⁺ influx through TRPCs, transiently increased Lp by 1.2-fold (from 1.6 ± 0.8 to 9.8 ± 2.7 × 10⁻⁶ cm sec⁻¹ cmH₂O⁻¹; P < 0.0001; n = 18). Protein kinase C inhibition by bisindolylmaleimide (1 μM) did not affect the OAG-induced increases in Lp. OAG also significantly increased microvascular endothelial [Ca²⁺]i, in vivo (n = 13; P < 0.0001), which again was not sensitive to protein kinase C inhibition. VEGF induced a transient increase in endothelial [Ca²⁺]i in human embryonic kidney cells (HEK-293) that were cotransfected with VEGF receptor 2 (VEGFR-2, also known as flk-1 or KDR), permeability; intracellular concentration; endothelium

The growth of new blood vessels in both physiological and pathological states is associated with increased vascular permeability (27). It has been hypothesized (9) that this increase in vascular permeability is necessary for the subsequent formation of proangiogenic extracellular matrix and hence subsequent angiogenesis. In recent years, some of the mechanisms through which vascular growth factors such as vascular endothelial growth factor (VEGF) act to increase permeability both acutely over a period of 2–3 min (1, 36) and chronically over hours to days (1) have been elucidated. It has been shown that VEGF receptor 2 (VEGFR-2, also known as flk-1 or KDR), when stimulated by VEGF, binds to and activates phospholipase C-γ (PLC-γ; Ref. 32) and that the VEGF-mediated increase in permeability is attenuated by PLC inhibitors (25). PLC generates inositol (1,4,5)-trisphosphate (IP₃) and diacylglycerol (DAG). IP₃ binds to receptors that gate Ca²⁺ release from Ca²⁺ stores in the endoplasmic reticulum. VEGF has been shown to increase endothelial intracellular Ca²⁺ concentration ([Ca²⁺]i) in vitro (3) and in vivo (2). Interestingly, however, this increase in endothelial [Ca²⁺]i is not affected by store depletion with sarco(endo)plasmic reticulum Ca²⁺-ATPase inhibitors such as thapsigargin (26). Inflammatory agents such as ATP have also been shown to increase endothelial [Ca²⁺]i, in vivo (12), and this Ca²⁺ increase is also linked to an increase in microvascular permeability (14). However, such G protein-coupled increases in endothelial [Ca²⁺]i, are inhibited by thapsigargin, which suggests that VEGF and ATP act through different Ca²⁺-influx pathways. Presumably ATP activates a store-operated Ca²⁺ channel (SOC), whereas VEGF is able to increase Ca²⁺ through a store-independent route. Because VEGF does not increase endothelial [Ca²⁺]i, from stores but does act through PLC-γ activation, it is likely that VEGF acts either via IP₃-mediated activation of thapsigargin-insensitive stores, through direct activation of IP₃-sensitive plasmalemmal Ca²⁺ channels, or by DAG-mediated pathways. We have previously shown that inhibition of protein kinase C (PKC) failed to affect VEGF-induced increases in microvascular permeability (25). In addition, inhibiting DAG lipase while failing to block the response to VEGF itself causes a transient permeability increase (25). These results together suggest that VEGF may act through PLC-mediated production of DAG to increase hydraulic conductivity (Lp) and endothelial [Ca²⁺]i.

DAG-activated Ca²⁺-influx channels have recently been described. These channels are members of a family of nonselective cation channels termed canonical (or classic) transient receptor potential channel (TRPC) proteins (5). Certain members of this family (specifically, TRPC-3, -6, and -7) can be directly stimulated by the membrane-permeant analog of DAG, 1-oleoyl-2-acetyl-sn-glycerol (OAG; Ref. 16). These three channels have varying levels of store dependence: TRPC-3 and -7 appear to be store independent. Furthermore, TRPC-3 and -6 have recently been described in microvascular endothelial cells (29). Although there are no specific inhibitors of the TRPCs, the recent discovery that flufenamic acid (FFA) can inhibit cation currents through TRPC-3 and -7 but stimulate those through TRPC-6 enables some initial pharmacological dissection of the signaling pathway. Therefore, if VEGF resulted in DAG production that directly stimulated TRPCs to stimulate Ca²⁺ influx in endothelial cells in vivo, then the
VEGF response and its resistance to PKC inhibition should be mimicked by DAG analogs. We have therefore measured the effects of OAG on \(L_p\) and \([Ca^{2+}]_i\), determined whether VEGF can stimulate TRPC-6 in a heterologous expression system, and measured the effects of an agent that differentially inhibits TRPCs.

**METHODS**

**Materials**

*In vivo measurement of \(L_p\) and \([Ca^{2+}]_i\).* All experiments were carried out on male leopard frogs, *Rana temporaria* or *Rana pipiens* (body wt, 20–35 g), which were supplied by Blades. All chemicals were purchased from Sigma except bisindolylmaleimide (BIM), which was purchased from Calbiochem. OAG was made up in ethanol (50 mM) or DMSO (100 mM), and BIM was made in DMSO (10 mM). VEGF was a kind gift of N. Ferrara (Genentech).

**Animal Procedures**

Frogs were anesthetized by immersion in 1 mg/ml 3-aminobenzoic acid ethyl ester (MS222) in water, and anesthesia was maintained by superfusion of the gut with 0.25 mg/ml MS222 in frog Ringer solution, which was composed of (in mM) 111 NaCl, 2.4 KCl, 1 MgCl\(_2\), 1.1 CaCl\(_2\), 0.2 NaHCO\(_3\), 5.1 D-glucose, 2.63 HEPES acid, and 2.37 HEPES sodium salt adjusted to pH 7.40 ± 0.02 with 0.115 mM NaOH. At the end of the experiments, frogs were humanely killed by destruction of the brain while still under anesthesia. Rats were anesthetized with 5% halothane by inhalation and were humanely killed by cervical dislocation before they regained consciousness. All experiments were carried out in conformance with Home Office guidelines.

**Measurement of \(L_p\)**

Anesthetized animals were laid supine, and the limbs were secured lightly to a supporting tray. 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 Perspex pillar. The microvessels in the mesentery were visualized under a Leitz (DMIL) inverted microscope. A video camera (WVBP32, 8 mm, Panasonic) was attached to the top of the microscope to allow binocular visualization and simultaneous recording of a 270-μm segment of the vessel (from a total length of 800–2,000 μm). The video was connected through an electronic timer (ForA Figure 1) and adapted to measure rapid and short-lasting changes in \(L_p\) (10). Microvessels were selected that had brisk blood flow with no white blood cells adhering to or rolling along the wall, at least 800 μm in length with no side branches, and a baseline \(L_p\) value of \(<10 \times 10^{-7}\) cm/s−1 cmH\(_2\)O−1. Microvessels chosen for \(L_p\) measurement were either true capillaries (divergent flow at one end and convergent flow at the other) or first-order venules (convergent flow from two true capillaries at one end and convergent flow at the other) and had diameter measurements of 15–35 μm. The vessel was cannulated with a glass micropipette [pulled and beveled to a fine hypodermic-style tip from glass capillary tubes (1.5 mm OD, Clark ElectroMed)] and filled with 1% BSA in frog Ringer solution and rat red blood cells as flow markers. The red rat blood cells were collected by direct cardiac puncture of halothane-anesthetized rats (5% halothane) and were washed three times in frog Ringer solution before use. The micropipette was clamped in a holder (WPI; Stevenage, UK) and connected to a water manometer and syringe refiller (15). Baseline \(L_p\) values were measured by occluding each vessel with a glass rod for 3–7 s while it was perfused with 1% BSA at a pressure of 30 cmH\(_2\)O. The vessel was then allowed to flow freely for at least 7 s before another occlusion was made.

**Determination of \(L_p\).** The transcapillary water flow per unit area of capillary wall (\(J/JA\)) was calculated from the initial velocity of the red blood cells (\(d/dt\)) after occlusion, the capillary radius (\(r\)), and the length between the marker cell and the point of occlusion (\(l\)), all of which were measured off-line from the videotape

\[
(J/JA) = (d/dt)/[r(l/2)]
\]

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

\[
L_p = (J/JA)/\Delta P
\]

where \(\Delta P\) is the effective hydrostatic and oncotic pressure difference between the capillary and the interstitium. The capillary pressure was set at 30 cmH\(_2\)O, so \(\Delta P\) was 26.4 cmH\(_2\)O (1% BSA has an effective oncotic pressure of 3.6 cmH\(_2\)O) assuming tissue pressure was negligible, and tissue oncotic pressure was equivalent to that in the superfusate (zero).

**Measurement of \(L_p\) during perfusion with agonists.** After baseline \(L_p\) measurement, the micropipette was refilled with a solution that contained 100 μM OAG or 1 nM VEGF. Refilling was observed as a change in the hematocrit of the perfusate, and the vessel was occluded for 3–5 s as soon as possible to measure \(L_p\). The occlusion was released and \(L_p\) was measured approximately every 10 s over the next 2–5 min. In experiments to measure the effects of the inhibitors BIM and FFA on OAG- or VEGF-induced changes in \(L_p\), respectively, the vessel was first perfused with 1 μM BIM or 100 μM FFA for 10 min before the pipette was refilled with a combination of 1 μM BIM and 100 μM OAG or 100 μM FFA and 1 nM VEGF. For BIM-OAG experiments, the vessel was washed out with BIM-free BSA-Ringer for 20 min and then perfused with OAG alone. For BIM-OAG experiments, vessels were only analyzed in which OAG alone resulted in an increase in permeability. We have previously shown that BIM blocks PKC activation in frog tissue (25), and others have used FFA in amphibian preparations (34). All perfusates were made up in 1% BSA in frog Ringer and contained rat red blood cells as flow markers.

**Measurement of \([Ca^{2+}]_i\)**

Changes in endothelial \([Ca^{2+}]_i\), were estimated in frog mesenteric microvessels as described previously (2, 13). The frog was anesthetized (as before) and laid supine, and the limbs were secured to a supporting tray. The gut was exposed and the exteriorized mesentery gently stretched over a coverslip and pinned to a Sylgard layer glued to the edge of the coverslip. The tray was placed on an extended microscope stage with the mesentery over a ×40 Fluorot objective (numerical aperture, 0.75) on an inverted epifluorescence microscope (Leica DMIRB) fitted with a 400-nm dichroic filter, and the mesentery was continuously superfused with frog Ringer solution (20–22°C) to visualize the mesenteric microvessels. A straight free-flowing capillary or postcapillary venule, 20–35 μm in diameter and free of white blood cells, was cannulated using a beveled glass micropipette that was bent by ~45–60° at the shoulder. The vessel was perfused with 5–10 μM fura-2 AM in 1% BSA (in frog Ringer solution at pH 7.4) at 30 cmH\(_2\)O. The vessel was illuminated by a high-intensity xenon arc lamp light source (Cairn Instruments). A rotating (50 Hz) disc that contained 340-, 360-, and 380-nm excitation filters was used to control the excitation wavelength. Incident white light was passed through an infrared (IR) filter (750 nm long pass) to visualize the preparation with an IR camera (WAT-902B, Watec). A second di-
chronic filter was placed in front of the photometer to reflect light <700 nm wavelength to the photomultiplier tube. Light of >700 nm wavelength passed through the dichroic filter to the IR camera. The photomultiplier tube and filter wheel were controlled by a Cairn spectrophotometer connected to a PowerLab/4SP (ADInstruments) system.

Loading of endothelial cells was monitored at 10-min intervals by measuring fluorescence intensity (IF) within a defined window downstream of the cannula site at wavelengths of 340, 380, and 360 nm (F340, F380, and F360, respectively) excitation and 510 nm emission and also by visual examination. The vessel was checked visually at 10-min intervals to ensure even loading. Vessels generally took 60–90 min to load. Successfully loaded vessels had a diffuse fluorescence throughout the endothelial cells with more intense fluorescence around the nuclei (due to increased cell thickness around the nucleus), and F380 values were 4-to-10-fold higher than background. Once loading was complete, the pipette was refilled with 1% BSA that contained a low hematocrit of washed rat erythrocytes (as flow markers to ensure continuous perfusion) for calculation of the baseline ratio.

Effects of OAG on [Ca2+]i

After baseline IF measurements were made, the vessel was perfused with 100 μM OAG in 1% BSA for 10 min and then by 10 μM ionomycin for 10 min as a positive control. During perfusion with agonists, IF measurements were made continuously at 0.25-s intervals. The effects of PKC inhibition on OAG responses were measured by perfusing the vessel with 1 μM BIM for 10 min and measuring IF perfusing for 10 min with a combination of 1 μM BIM and 100 μM OAG, and then repeating the procedure in the absence of BIM. After ionomycin perfusion, the vessel was perfused with 1% BSA that contained a combination of 5 mM MnCl2 and 10 μM ionomycin. This final perfusion quenched the fluorescence from the Ca2+-sensitive form of fura-2 AM to yield an accurate background-intensity reading. All IF values were measured during perfusion at 30 cmH2O.

Calculation of [Ca2+]i

The ratio of IF340 to IF380 (IF340/IF380) is reported as an indication of changes in [Ca2+]i.

\[
[Ca^{2+}]_i = K \times \left[ \frac{R - 0.85R_{\text{max}}}{0.85R_{\text{max}} - R} \right]
\]

where R is the normalized ratio of IFi calculated as R = IFmax/IFmin; Rmin = (IF340 – B340)/(IF380 – B380); IF340 and IF380 are the fluorescence intensities with excitation at 340 and 380 nm, respectively; B340 and B380 are the background fluorescence intensities at excitation of 340 and 380 nm, respectively (measured as IFi before loading or, for Ca2+ calculation, after Mn2+ quenching); Rmax is the in vitro ratio for zero [Ca2+]; Rmax is the in vitro ratio at saturating Ca2+; and K is the product of the effective dissociation constant for fura-2 AM and the in vitro fluorescence intensity ratio of zero to saturating Ca2+. Owing to the technical difficulty inherent in these types of experiments, a background quench was not always possible; therefore, values are occasionally given as ratios with B340 and B380 measured as IF before perfusion with 1% BSA before loading with fura-2 AM.

Measurement of [Ca2+]i, in Heterologous Expression System

Human embryonic kidney (HEK) 293 cells were grown to 50–90% confluence in 60-mm-diameter dishes. Cells were transfected using Lipofectamine Plus (GIBCO-BRL). The following plasmids were used: mouse VEGFR-2 cDNA (Ik-1) cloned into the expression vector pBJ (a kind gift from Tim Quinn, University of California-San Francisco), human TRPC-6 cDNA cloned into the expression vector pcDNA3 (kind gift of Thomas Gudermann, Institute for Pharmacology, Berlin, Germany), and peq176, a plasmid that contains the cDNA for β-galactosidase (a kind gift from Michael Hanley, University of California-Davis). The cells were transfected with either VEGFR-2, TRPC-6, TRPC-6 and VEGFR-2, or peq176. Transfection efficiency was assessed by staining peq176-transfected cells for β-galactosidase. Expression of VEGFR-2 and TRPC-6 was confirmed by RT-PCR of mRNA extracted from cells not used for Ca2+ measurement. The peq176-transfected cells were fixed with 4% formaldehyde in PBS, stained with X-gal staining solution that contained 4 mM MgCl2, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, and 67 μM X-gal (Roche Diagnostics) in PBS. Cells positively transfected with β-galactosidase stained a bright blue, which gave an indication of the percentage of cells transfected in each treatment.

Transfected cells were washed once with 0.5% EDTA-1× PBS and twice with 1× PBS, and then cells were washed off the dishes and divided between coverslips (Chance Proper) in six-well plates (Nalge Nunc, Fisher). Cells were loaded 48 h later with 10 μM fura-2 AM diluted in phenol red-free DMEM (GIBCO-BRL) that contained 4.8 μM Phromic acid (Molecular Probes) for ≤2 h. The cells were then incubated in HBSS (GIBCO-BRL), and [Ca2+]i values were determined via the IF measurements of fura-2 AM-loaded cells by excitation at 340 and 380 nm and recording emission at 510 nm. The calculated 340-to-380-nm ratio (Rnorm) is proportional to [Ca2+]i, as discussed earlier. Cells were then treated with 1 nM VEGF in HBSS, and the fluorescence ratio was measured. The cells transfected with p eq176 were used as a negative transfection control. Fluorescence ratio was measured on the same system as for the in vivo measurement of [Ca2+]2, using an adapter to accept coverslips.

RT-PCR

Mesenteric microvessels (50–100 μm diameter) were microdissected from male Wistar rats that were killed by cervical dislocation. The mRNA was extracted from these vessels using standard procedures (4). Reverse transcription was carried out using 1 μg of RNA and 5 μl oligo(dT) (Promega) in 10 μl of RNase-free water (Sigma). This mixture was incubated at 65°C for 5 min and was immediately placed on ice. The reaction mixture was altered to 1× first-strand synthesis buffer (Roche) composed of 10 mM DTT (Roche), 2.5 mM dNTP (Promega), 1 unit of RNA guard (Amersham), and 2.5 units of Expand RT (Roche) in a total of 20 μl of RNase-free water (Sigma). This was incubated at 42°C for 2 h. PCR was performed using the primers listed in Table 1. The PCR mixture consisted of 1× PCR buffer (Abgene), 1.25 mM MgCl2 (Abgene), 375 mM dNTP, 10 μM forward primer, 10 μM reverse primer (except for GAPDH, where 5 μM of each primer was used), 1 μl of cDNA, and 1 unit of Taq (Abgene) in 20 μl of RNase-free water. A standard PCR cycle was used, i.e., 35 cycles of 55°C annealing, 72°C extension, and 96°C denaturing for 30 s each (PCR Express, Hybaid). RT-PCR products were run on 2% agarose (Roche) gels in the presence of 0.5 μg/ml ethidium bromide (Invitrogen). Gels were photographed under ultraviolet transillumination (GIBCO-BRL). A 100-bp ladder (Sigma) was used to visualize bands from 100–1,000 bp.

Table 1. Primers used for PCR

<table>
<thead>
<tr>
<th>TRPC</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
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<tbody>
<tr>
<td>TRPC-1</td>
<td>5’-CCCTGAAGGTGTTGCTGACA-3’</td>
<td>5’-TGGCTTGCAGAAGATGCC-3’</td>
</tr>
<tr>
<td>TRPC-3</td>
<td>5’-ATGGATCCTTCTTCTACTACGTTGGG-3’</td>
<td>5’-GAAGCTTGTCAAGCCAAATCCAGGTTGC-3’</td>
</tr>
<tr>
<td>TRPC-4</td>
<td>5’-TTCGATCTCAGCGCTTGGTCGTT-3’</td>
<td>5’-TGCTGTTCACAGAAGATGCC-3’</td>
</tr>
<tr>
<td>TRPC-6</td>
<td>5’-GGGAAAGATTAAGGTGGGC-3’</td>
<td>5’-CGAAAAATGCATGATCTCG-3’</td>
</tr>
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TRPC, transient receptor potential channel.

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Statistics

An \( L_p \) response was defined as an increase to a peak that was at least two standard deviations higher than the mean baseline (taken from at least three occlusions during BSA perfusion; Ref. 1). Measurements of \( L_p \) from control vessels have previously been shown to be non-normally distributed (11). Therefore, nonparametric statistics were used to compare and contrast \( L_p \) values [median ± interquartile range (IQR), Wilcoxon for paired and Mann-Whitney U for unpaired tests]. Normalized increases in \( L_p \) were expressed as means ± SE. IQR ratios were compared using parametric tests. Responses to VEGF were compared with baseline values using the paired \( t \)-test, and responses to VEGF were compared between different transfections using an unequal sample variance \( t \)-test. A probability value \( P < 0.05 \) was accepted as statistically significant.

RESULTS

Effects of OAG on \( L_p \)

Measurements were made on 32 vessels perfused with 100 \( \mu \)M OAG. Of these vessels, only 18 responded to the agonist. Four examples of permeability measurements are shown in Fig. 1. Perfusion with OAG typically caused a rapid, transient increase in permeability that returned to control values within 3 min. In these 18 vessels, perfusion with OAG increased \( L_p \) from a baseline value of 1.6 ± 0.8 \( \times \) \( 10^{-7} \) cm \( \cdot \) s \(^{-1} \) \( \cdot \) cmH \(_2\)O \(^{-1} \) (median ± IQR) to a peak of 9.8 ± 2.7 \( \times \) \( 10^{-7} \) cm \( \cdot \) s \(^{-1} \) \( \cdot \) cmH \(_2\)O \(^{-1} \) (\( P < 0.0001 \)). \( L_p \) returned to baseline within 3 min in all 18 vessels.

Effects of BIM on OAG-Induced \( L_p \)

Perfusion of vessels with BIM did not affect the OAG-mediated response. In seven vessels in which OAG subsequently yielded an increase in \( L_p \), 1 \( \mu \)M BIM was perfused for 10 min before addition of OAG. This did not affect the baseline \( L_p \) value. Examples of permeability measured in a single vessel (Fig. 2A) or in two different vessels with the same baseline permeability (Fig. 2B) are shown. Perfusion with BIM and OAG together increased \( L_p \) in the same manner as that with OAG alone. The time course of the increase averaged over all 7 vessels (BIM + OAG) or all 18 vessels (OAG alone) is shown in Fig. 2C. In all seven vessels perfused with OAG and BIM, the \( L_p \) increased 2.5 ± 0.6-fold (Fig. 2C) from a median baseline of 3.5 ± 1.3 \( \times \) \( 10^{-7} \) cm \( \cdot \) s \(^{-1} \) \( \cdot \) cmH \(_2\)O \(^{-1} \) to a median peak of 9.5 ± 1.5 \( \times \) \( 10^{-7} \) cm \( \cdot \) s \(^{-1} \) \( \cdot \) cmH \(_2\)O \(^{-1} \) (Fig. 2D; \( P < 0.05 \)). This was not significantly different from that in the 18 vessels with OAG alone (a 3.8 ± 1.2-fold increase; Fig. 2D) or from the increase in \( L_p \) induced by OAG alone in those 7 vessels in which \( L_p \) was measured with BIM perfusion (baseline 1.8 ± 0.7 \( \times \) \( 10^{-7} \) cm \( \cdot \) s \(^{-1} \) \( \cdot \) cmH \(_2\)O \(^{-1} \) to a median peak of 9.1 ± 4.4 \( \times \) \( 10^{-7} \) cm \( \cdot \) s \(^{-1} \) \( \cdot \) cmH \(_2\)O \(^{-1} \), \( P < 0.05 \); 2.9 ± 0.6-fold).

Effects of OAG and VEGF on Same Vessels

Approximately half of the vessels responded to OAG. Interestingly, previous studies in this laboratory have shown that
50% of vessels respond to VEGF. If VEGF acts through DAG, then one might predict that OAG should increase the permeability in vessels in which VEGF can increase permeability. Conversely, if vessels that respond to VEGF do not respond to OAG, this would suggest that VEGF does not act via DAG production. To answer this question, six vessels were perfused with VEGF and permeability was measured, and the vessels were washed out with 1% BSA and then perfused with 100 μM OAG before permeability was measured again. In four vessels (Fig. 3A) a response to VEGF was seen, and in all four of these
vessels a response to OAG was also seen. In two vessels there was no response to either VEGF or OAG. Because in previous experiments in this laboratory VEGF responses have been seen in 50% of the vessels, the probability of six vessels showing the same response to VEGF and OAG is therefore 0.016, i.e., highly significant. To our surprise, there was a highly significant correlation between the size of the response with OAG and that with VEGF (Fig. 3B, $r = 0.94; P < 0.02$, Spearman rank correlation coefficient). In two vessels it was possible to measure $[\text{Ca}^{2+}]_i$ first with OAG and then with VEGF. In one case, OAG increased $[\text{Ca}^{2+}]_i$ from 86 to 147 nM and VEGF also increased $[\text{Ca}^{2+}]_i$, with a similar time course, from 88 to 193 nM. In the other case, OAG increased $[\text{Ca}^{2+}]_i$ from 77 to 141 nM and VEGF increased $[\text{Ca}^{2+}]_i$ from 56 to 176 nM.

**Effects of OAG and BIM on $[\text{Ca}^{2+}]_i$**

Measurements of $[\text{Ca}^{2+}]_i$ were made on 27 vessels perfused with 100 μM OAG. Of these vessels, 18 responded to the agonist. Perfusion with OAG caused an increase in $[\text{Ca}^{2+}]_i$ that peaked after 2–5 min (Fig. 4A). In five vessels, a measurement after quenching was not successfully made, so these were excluded from further analysis. Perfusion of vessels with BSA or vehicle (DMSO) did not result in any change in ratio even after 30 min of perfusion. In 13 vessels, the $[\text{Ca}^{2+}]_i$ increased from a baseline of 59 ± 6.5 nM to a peak of 155 ± 18 nM (means ± SE, $n = 13; P < 0.001$; Fig. 4C). To determine whether OAG was acting through PKC, the PKC inhibitor BIM was used. OAG perfusion was preceded by perfusion first with
VEGF increased $[\text{Ca}^{2+}]$ and $L_p$ through PLC activation (25) independently of IP$_3$-mediated store release (26) or PKC- or DAG lipase-mediated pathways (25). Because DAG has been shown to directly activate transient receptor potential cation channels (16), we tested the hypothesis that VEGF increases $[\text{Ca}^{2+}]$, through activation of a TRPC in a heterologous expression system. HEK-293 cells were transfected with either the mouse VEGFR-2 cDNA, the human TRPC-6 cation channel cDNA, a control vector (peq176, which contains the β-galactosidase cDNA, used to ensure that transfection efficiency was $>80\%$), or both VEGFR-2 and TRPC-6 cDNAs. Figure 5 shows the effects on the $I_{F_{340}}/I_{F_{380}}$ ratio of adding $1 \text{nM VEGF}$ to some of these transfected cell types. Addition of $1 \text{nM VEGF}$ to cells transfected with the control vector, VEGFR-2, or TRPC-6 alone did not show any increase in the $I_{F_{340}}/I_{F_{380}}$ ratio (Fig. 5, A–C). Addition of VEGF to cells cotransfected with VEGF and TRPC-6, however, resulted in a robust, transient increase in the $I_{F_{340}}/I_{F_{380}}$ ratio (Fig. 5D) similar to that revealed by VEGF in endothelial cells in vivo. In 11 repeated experiments, the $I_{F_{340}}/I_{F_{380}}$ ratio increased from $2.87 \pm 0.40$ to $3.18 \pm 0.42$ ($28 \pm 3.8\%$, Fig. 5E; $P < 0.05$, paired t-test). There was no increase in the ratio measured during addition of VEGF to control transfected cells (from $4.69 \pm 0.46$ to $3.82 \pm 0.43$; $P > 0.05$), TRPC-6 transfected cells (from $2.32 \pm 0.06$ to $2.56 \pm 0.24$; $P > 0.05$), or from VEGFR-2-transfected cells ($1.75 \pm 0.29$ to $1.72 \pm 0.31$).

Effects of VEGF on $[\text{Ca}^{2+}]$ in Cells Transfected with TRPC-6

To determine whether modulators of TRPCs were able to alter the response to VEGF, we measured the effects of the TRPC modulator FFA. This compound has been shown to enhance the conductance of TRPC-6 and block other DAG-sensitive TRPCs such as TRPC-3 or -7 (17, 18) among a number of other properties. Perfusion of the vessels with FFA resulted in variable effects on the response to VEGF. Figure 6A shows the effects in a single vessel of FFA perfusion. VEGF alone resulted in a significant increase in $L_p$ from $0.5$ to $11.8 \times 10^{-7} \text{cm} \text{s}^{-1} \text{cmH}_2\text{O}^{-1}$, and a $20\text{-min}$ washout with $1\%$ BSA and $10\text{ min}$ of perfusion with $100 \mu\text{M FFA}$, VEGF only increased $L_p$ from $0.7$ to $1.5 \times 10^{-7} \text{cm} \text{s}^{-1} \text{cmH}_2\text{O}^{-1}$, i.e., a significant inhibition. Figure 6B shows the effects of FFA in a different vessel from a different animal. In this vessel, VEGF increased $L_p$ only slightly, from $0.8$ to $1.7 \times 10^{-7} \text{cm} \text{s}^{-1} \text{cmH}_2\text{O}^{-1}$. However, after $10 \text{ min}$ of perfusion with $100 \mu\text{M FFA}$, VEGF increased $L_p$ from $1.9$ to $10.0 \times 10^{-7} \text{cm} \text{s}^{-1} \text{cmH}_2\text{O}^{-1}$. This pattern was typical; approximately half of the vessels responded to FFA by inhibiting and the other half by enhancing the effects of VEGF. Overall, in 11 vessels, 5 vessels yielded a response to VEGF that was inhibited by FFA, and 6 yielded a response to VEGF that was enhanced by FFA. Therefore, although overall there was a slight and not significant inhibition of the VEGF response by FFA (Fig. 7A), differential analysis of the responses shows that FFA can enhance (Fig. 7B) or inhibit (Fig. 7C) the response to VEGF. This is unusual in that VEGF usually increases $L_p$ by a similar magnitude on successive applications (1). Therefore, we differentially analyzed the vessels according to whether the re-

BIM (1 μM) and then with BIM and OAG together. Vessels that did not respond to OAG alone were excluded from further analysis. In five vessels, a similar increase in $[\text{Ca}^{2+}]$ was observed in the presence of BIM to that in vessels not exposed to BIM: the ratio increased from $1.50 \pm 0.19$ to $2.40 \pm 0.36$ ($n = 5$; $P < 0.05$). However, for technical reasons, the $[\text{Ca}^{2+}]$ could only be calculated in three of these vessels. OAG increased $[\text{Ca}^{2+}]$, in the presence of 1 μM BIM from $53 \pm 12$ to $201 \pm 49$ nM (Fig. 4, B and C), which was not different from the increase in the absence of BIM (from $51 \pm 19$ to $196 \pm 58$ nM in those three vessels).
response to VEGF in the presence of FFA was greater (enhancers) or reduced (inhibitors) as shown in Fig. 7, B and C, respectively. The mean baseline \( L_p \) value (during perfusion with BSA or FFA) was significantly lower in vessels in which FFA had an inhibitory effect (Fig. 8A). Furthermore, in vessels in which VEGF caused a very weak increase in \( L_p \), FFA perfusion caused a significant enhancement of the response (Fig. 8B). However, this was not simply an effect of the increase in baseline used to calculate the fold increase, because the fold increase in the enhanced responding vessels was greater than that in the inhibitory responding vessels (\( P < 0.02 \); Fig. 8C). The relative response of the vessel to VEGF with FFA to that with VEGF alone was compared with the relative response of 18 vessels in which VEGF was given twice (2, 25, 35). The logarithm of the relative value (i.e., the fold increase with FFA divided by the fold increase without FFA) would be zero if the two responses were identical. Figure 8D shows that in vessels given a second application of VEGF, the log of the relative response varied around zero from \(-0.30\) to \(+0.38\) (i.e., \(50\%\)–\(244\%\)). FFA treatment resulted in a significant increase in variance of the VEGF response (\( P < 0.001\), F-test) from \(-1.0\) to 0.72.
(10–520%). In three vessels, niflumic acid (100 μM) did not affect the VEGF response.

Expression of TRPCs in Rat Microvasculature

The TRPC sequences have not yet been described in Rana species. However, this typical transient Lp and solute permeability response to VEGF has previously been shown to occur in rat mesenteric and coronary microvessels, respectively (21, 36). Therefore, to determine which TRPCs could be responsible for the increase in permeability in rat microvessels, mRNA was extracted from 100 to 200-μm-diameter rat mesenteric microvessels. Expression of TRPC-4 and -6 but not TRPC-3 or -1 was detected by RT-PCR (Fig. 9).

DISCUSSION

In this study, we showed that the DAG analog OAG transiently increases the Lp of frog mesenteric microvessels with a time course similar to that of VEGF. As far as we are aware, this is the first time that DAG analogs have been shown to increase vascular permeability. When VEGF was first isolated, it was assumed (because it had been shown to activate PLC; Ref. 10) that this would increase permeability by production of IP₃, which would lead to release of Ca²⁺ from intracellular stores and a subsequent Ca²⁺-dependent Ca²⁺ influx. There was subsequent indirect evidence for this in in vitro studies that showed that VEGF resulted in IP₃ production (22) but there was no direct evidence of the effect of VEGF on Ca²⁺ stores. This hypothesis was thrown into doubt by the discovery that VEGF increases Lp through a Ca²⁺-dependent mechanism (2) that is not sensitive to thapsigargin-induced store depletion (26). It is therefore possible that VEGF signals through a DAG-dependent pathway and not an IP₃-mediated one.

OAG is commonly used to mimic DAG, as it is freely permeable across the cell membrane. The finding that OAG alone increases Lp strongly implicates DAG as a candidate for mediating permeability changes in endothelial cells. This suggestion is supported by results from our previous study (25),

Fig. 6. Effects of flufenamic acid (FFA) on VEGF-mediated increase in single capillaries. Effects of perfusion with 1 nM VEGF or 1 nM VEGF and 100 μM FFA are shown for two vessels. A: Lp was significantly and transiently increased by perfusion with 1 nM VEGF. However, after washout, recovery, and preincubation with FFA, VEGF did not increase Lp in the presence of FFA. B: the reverse was true, in that with VEGF alone there was only a small increase in Lp, but in the presence of FFA, a large, transient increase in Lp was measured.

Fig. 7. Effects of FFA on VEGF-mediated Lp increases. A: time course of the mean fold increase in Lp for all 11 vessels perfused with 1 nM VEGF or 1 nM VEGF and 100 μM FFA. There appeared to be a small reduction in the size of the response when all 11 vessels were averaged. However, when the experiments are divided into those where FFA enhanced (B) or inhibited (C), it can be seen that there are clearly two different populations of vessels.
which showed that inhibition of DAG lipase (a major pathway for the metabolism of DAG) causes an increase in $L_p$. We have previously shown that VEGF increases both $\text{Ca}^{2+}$ and permeability in approximately half of perfused vessels (1, 2). We have assumed (although without direct evidence) that this heterogeneity was due to differences in VEGF receptor expression between vessels. The fact that 14 out of 32 vessels studied in this series of experiments did not respond to OAG indicates that the heterogeneity may also be downstream of the receptor and that some vessels contain endothelial cells that do not respond to DAG analogs; i.e., they do not contain the downstream signaling mechanisms such as DAG-sensitive ion channels.

If DAG directly increases microvascular permeability through activation of TRPCs, then this increase is likely to be associated with an increase in endothelial $[\text{Ca}^{2+}]_i$ in these vessels. Indeed, OAG stimulates a significant increase in $[\text{Ca}^{2+}]_i$, which was independent of PKC activation. The inhibitor BIM was used because it is a competitive inhibitor of the ATP-binding site of PKC (30) and does not interfere with the DAG-binding site. This is important, because inhibitors of the DAG-binding site may also affect DAG binding to other proteins such as the TRPCs. It is of note, however, that the OAG $\text{Ca}^{2+}$ response was slower than the permeability response. This is also true of the $\text{Ca}^{2+}$ responses to ATP (14), ionomycin (13), and VEGF (2). It is possible that the size and timing of the $L_p$ response is not correlated with the time taken to reach a peak $\text{Ca}^{2+}$ concentration, but rather is correlated with the time taken to reach a maximum rate of increase of $[\text{Ca}^{2+}]_i$ in the cells, or the global $[\text{Ca}^{2+}]_i$ measured in this system may not reflect the local $[\text{Ca}^{2+}]_i$ changes that actually regulate $L_p$.

Three members of the TRPC family, TRPC-6, -3, and -7, have been shown to gate OAG-induced $\text{Ca}^{2+}$ influx in heterologous expression systems (16) and in PC12 cells, which is an adrenal chromaffin cell line that endogenously expresses the channel (28). This latter OAG-stimulated $\text{Ca}^{2+}$ influx occurred even after maximal activation by acetylcholine. These channels were inhibited by SKF-96365 and FFA (blockers of nonselective cation channels) but not by 2-aminoethoxyphenyl borate, an IP3 receptor inhibitor, which suggests that they were not coupled to IP3 receptor activation. Interestingly, the VEGF-mediated increase in permeability is also blocked by SKF-96365 and FFA but not by store depletion with thapsigargin. Independence of TRPC-3 from IP3 receptor activation has been demonstrated in that PLC activation in IP3 receptor-deficient cell lines (31) still activated TRPC-3. Hofmann et al. (16) demonstrated that OAG-induced $\text{Ca}^{2+}$ currents in TRPC-6-
expressing (CHO-K1) cells were not sensitive to PKC inhibition, which suggests that these channels were directly responsive to DAG. Again we have shown that OAG stimulates an increase in permeability that is temporally similar to the VEGF response and is not sensitive to PKC inhibition. Because there is significant evidence from studies with transfected cells that the TRPCs can mediate Ca\(^{2+}\) entry and are gated by DAG but are store and PKC independent (reviewed in Ref. 23), we sought to further test whether TRPCs were involved in the VEGF-mediated Ca\(^{2+}\) response.

We have shown here that cotransfection of HEK cells with VEGFR-2 and TRPC-6 enables the cells to respond to VEGF by increasing the [Ca\(^{2+}\)]\(_i\). There is one report that 293 cells transfected with VEGFR-2 alone can respond to VEGF by increasing Ca\(^{2+}\) concentration (33) in the absence of transfection with TRPCs. Interestingly, Wen et al. (33) reported this in single cells, since only 20% of the transfected cells responded to VEGF. In our experiments, we measured the overall Ca\(^{2+}\) response from a population of cells; therefore, any effect of VEGFR-2 transfection alone in a small subset of cells was probably not sufficient to record a change in Ca\(^{2+}\) in this system. It would be interesting to determine whether the proportion of HEK cells responding in the system described by Wen et al. was increased by transfection with TRPCs. As far as we are aware, these are the first experiments that describe a link between the VEGF receptors and the TRPCs. However, TRPCs have been implicated in other growth factor-mediated Ca\(^{2+}\)-signaling pathways, particularly the platelet-derived growth factor stimulation of vascular smooth muscle cells (18), and brain-derived neurotrophic factor activation of tyrosine protein kinase receptor TrkB in neurons (19). Both of these growth factors act through receptor tyrosine kinases, and the VEGF receptor is part of the platelet-derived growth factor receptor family. Therefore, the involvement of TRPCs in VEGF signaling is perhaps not so surprising.

The findings that the response to VEGF could be both inhibited and stimulated by FFA are wholly consistent with these findings. However, it is not possible to interpret these experiments as being more than consistent with this hypothesis, because FFA, aside from having differing effects on TRPCs [i.e., inhibition of TRPC-3 but enhancement of TRPC-6 (17)], is a very nonspecific inhibitor/stimulator of TRPCs. FFA is perhaps better known as a Cl\(^{-}\) channel inhibitor (7, 34), but it also potentiates large-conductance Ca\(^{2+}\)-dependent K\(^{+}\) channels (24) and inhibits gap junctions by its actions on connexins 50 and 46 (8). The fact that it can either potentiate or inhibit the VEGF response can be interpreted as evidence for a heterogeneity of TRPCs in vascular endothelium, but does not provide definitive proof of this. In the absence of specific pharmacological TRPC inhibitors, molecular genetic experiments may prove more enlightening. The variation in the effects of FFA from vessel to vessel are perhaps not surprising considering the heterogeneity of capillaries in a single vascular bed. Interestingly, it has recently been shown that there is enormous heterogeneity in the Ca\(^{2+}\) responses to agonists such as ATP even within an individual vessel (19a). This appears to be due to differences in the expression of Ca\(^{2+}\)-dependent K\(^{+}\) channels within these cells, although the principle would equally apply to TRPC channels. In summary, these experiments clearly demonstrate that membrane-permeant analogs of DAG stimulate a significant increase in vascular permeability independently of PKC in a similar manner to VEGF. Furthermore, OAG also stimulates increased [Ca\(^{2+}\)], in endothelial cells of microvessels in vivo.
again independently of PKC. We have described a link between VEGFR-2 signaling and activation of at least one transient receptor potential nonselective cation channel that allows Ca$^{2+}$ entry into transfected cells (TRPC-6), and we have shown that a compound that inhibits some TRPCs and stimulates others has opposing effects in differing vessels. The presence of an OAG-dependent, PKC-independent increase in Ca$^{2+}$ that can be mimicked by inhibitors of DAG lipase and is store independent is considered prima facie evidence for the involvement of a canonical TRPC of the TRPC-3, -6, or -7 family. These data taken together with previous studies on VEGF signaling in vivo lead us to propose that at least one mechanism of action of VEGF is that it increases [Ca$^{2+}$], through store-independent TRPCs and, in particular, TRPC-6.

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