Platelet-Derived Growth Factor Maintains Stored Calcium Through a Nonclustering Orai1 Mechanism But Evokes Clustering If the Endoplasmic Reticulum Is Stressed by Store Depletion

Lynn McKeown, Nicholas K. Moss, Paul Turner, Jing Li, Nikki Heath, Dermot Burke, David O’Regan, Mark S. Gilthorpe, Karen E. Porter, David J. Beech

Rationale: Calcium entry through Orai1 channels drives vascular smooth muscle cell migration and neointimal hyperplasia. The channels are activated by the important growth factor platelet-derived growth factor (PDGF). Channel activation is suggested to depend on store depletion, which redistributes and clusters stromal interaction molecule 1 (STIM1), which then coclusters and activates Orai1.

Objective: To determine the relevance of STIM1 and Orai1 redistribution in PDGF responses.

Methods and Results: Vascular smooth muscle cells were cultured from human saphenous vein. STIM1 and Orai1 were tagged with green and red fluorescent proteins to track them in live cells. Under basal conditions, the proteins were mobile but mostly independent of each other. Inhibition of sarco-endoplasmic reticulum calcium ATPase led to store depletion and dramatic redistribution of STIM1 and Orai1 into coclusters. PDGF did not evoke redistribution, even though it caused calcium release and Orai1-mediated calcium entry in the same time period. After chemical blockade of Orai1-mediated calcium entry, however, PDGF caused redistribution. Similarly, mutagenic disruption of calcium flux through Orai1 caused PDGF to evoke redistribution, showing that calcium flux through the wild-type channels had been filling the stores. Acidification of the extracellular medium to pH 6.4 caused inhibition of Orai1-mediated calcium entry and conferred capability for PDGF to evoke complete redistribution and coclustering.

Conclusions: The data suggest that PDGF has a nonclustering mechanism by which to activate Orai1 channels and maintain calcium stores replete. Redistribution and clustering become important, however, when the endoplasmic reticulum stress signal of store depletion arises, for example when acidosis inhibits Orai1 channels. (Circ Res. 2012;111:66-76.)

Key Words: calcium channel ■ calcium stores ■ vascular smooth muscle cells ■ growth factor ■ acidosis ■ endoplasmic reticulum stress ■ store depletion ■ vascular remodeling

In its contractile phenotype, the vascular smooth muscle cell (VSMC) in the medial layer of arteries and veins is important for vascular integrity and tone. During development, however, the VSMCs can also exist in a noncontracting phenotype that has proliferating and migrating properties, which are important for the generation of new blood vessels. Features of these modulated VSMCs are altered Ca$^{2+}$ entry and Ca$^{2+}$ uptake mechanisms. In the adult, VSMCs may also switch to this state to enable vascular remodeling. One of the drivers of the proliferating phenotype is platelet-derived growth factor BB (PDGF), a promigratory signaling peptide. In addition to its roles in physiology, PDGF contributes in cardiovascular diseases that include pulmonary arterial hypertension and restenosis.

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The signaling pathways downstream of PDGF were only recently recognized to include activation of a calcium entry channel generated by Orai1 proteins and involving the calcium-sensing regulatory protein stromal interaction mole-
Orai1 was found to be a positive modulator of VSMC migration, with knock-down of its expression suppressing neointimal formation after vascular injury. The mechanism by which PDGF activates Orai1 channels has not been extensively studied but the effect is suggested to occur via PDGFRβ and the downstream pathways of phospholipase C and Ca²⁺ release. Orai1 channels are activated by store depletion via a mechanism that involves sensing of luminal Ca²⁺ in the stores by STIM1 and then physical interaction of STIM1 with Orai1 channels in the plasma membrane. It has therefore been assumed that PDGF-evoked Ca²⁺ release leads to store depletion, which is sensed by STIM1, which then activates Orai1.

Orai1 was originally identified in the immune system. It is suggested that Orai1 provides the molecular basis for the ion pore-forming subunits of Ca²⁺-selective channels that are activated by store depletion and have commonly been referred to as CRAC channels. The mechanism proposed for activation of the channels by store depletion is striking. Activation ultimately involves physical interaction between STIM1 (of the stores) and Orai1 (of the plasma membrane), but first, there are major cellular redistributions of STIM1 and Orai1. Before store depletion, STIM1 is dynamically active as a partner of the microtubule binding protein EB1. The STIM1 has an N-terminus that is localized to the store lumen and contains an EF-hand with affinity for Ca²⁺. When the stores are full, STIM1 is bound to luminal Ca²⁺. However, depletion of Ca²⁺ in the stores leads to Ca²⁺ dissociation from STIM1 and a chain of events that ends with major redistribution of STIM1 from microtubules to static oligomerized focal clusters (puncta) under the plasma membrane. Simultaneous studies of clustering and Ca²⁺ influx have suggested that STIM1 translocation precedes Orai1 channel opening.

Each Orai1 protein is thought to contain 4 transmembrane segments with intracellular N- and C-termini. Channels are considered to arise from 4 Orai1 proteins assembled around a central Ca²⁺-selective pore. Before store depletion, the Orai1 channels are reasonably uniformly distributed across the cell surface. However, store depletion leads to Orai1 recruitment to sites of STIM1 clusters, such that the Orai1 and STIM1 become aggregated. An interaction domain in the cytosolic STIM1 C-terminus (the CAD or SOAR domain) then directly binds Orai1 to activate the channels and enable Ca²⁺ influx. Therefore, Orai1 activation is considered to require not only Ca²⁺ sensing by STIM1 but extensive redistribution and co-clustering.

The purpose of this investigation was to determine the relevance of the redistribution and clustering of Orai1 and STIM1 in the PDGF responsiveness of proliferating VSMCs. To achieve this objective, we used fluorescently tagged Orai1 and STIM1 so that subcellular dynamics of these proteins could be specifically tracked in real-time in living VSMCs.

## Methods

An expanded Methods section is available in the online Data Supplement.

### Cell Preparation and Culture

Freshly discarded human saphenous vein segments were obtained anonymously and with informed consent from patients undergoing open heart surgery. Approval was granted by the Leeds Teaching Hospitals Local Research Ethics Committee. Proliferating VSMCs were prepared, using an explant technique, and grown in Dulbecco’s Modified Eagle’s Medium supplemented with 10% fetal calf serum, penicillin/streptomycin, and L-glutamine at 37°C in a 5% CO₂ incubator. Experiments were performed on VSMCs passaged 3 to 5 times; all of the cells stained positively for smooth muscle α-actin and smooth muscle myosin heavy chain and were noncontractile.

### Molecular Biology

cDNA encoding wild-type mCherry-tagged Orai1 was from S. Muallim and cDNA encoding eYFP-STIM1 was from T. Meyer. mCherry-Orai1 R91W mutagenesis was performed using mutagenic primers and Phusion high-fidelity polymerase (NEB, Herts, UK) according to the manufacturer’s instructions. Full-length cDNA for Orai1 (BC013386) was purchased from Geneservice (Source Bioscience LifeSciences, Nottingham, UK) and sub cloned into pcDNA6/V5-His (Invitrogen, Paisley, United Kingdom) at EcoRI/XhoI sites. The hemagglutinin (HA) tag (YPYDVPDYA) was inserted into the S3-S4 linker between Lys214 and Ala220 using a PCR fusion protocol. The first PCR fragment was used to linearize Orai1 in pcDNA6 from Ala220 to Lys214 and insert a 3′ Gly, Ser linker (forward primer: 5′ gagctagggcgcagcc; reverse primer 5′ gttccctctgctgtgggct). The second PCR product amplified full-length HA with sequences overlapping the first PCR fragment (forward primer: 5′ caagggagaagggcgacatatat; reverse primer 5′ ttgacgtcctctgctgtgggct). The fragments were recombined using the BD In-fusion PCR cloning kit (Takara Bio Europe, Paris, France) as per the manufacturer’s instructions. A construct encoding enhanced green fluorescent protein (eGFP) fused to the C-terminus of Orai1-HA was prepared through PCR cloning as described above. Clones were sequenced to confirm accuracy and identity.

### Cell Transfection, Live Cell Imaging, and Image Analysis

VSMCs (0.5 to 2×10⁶) were centrifuged (100 g) for 5 minutes, resuspended in Basic Nucleofector solution (Amaxa GmbH, Köln, Germany), mixed with 1.5 μg eYFP-STIM1 and 0.5 μg mCherry-Orai1, and transferred into a cuvette for electroporation (Amxaza). Cells were transferred from cuvettes to prewarmed culture medium and incubated in a 5% CO₂ incubator at 37°C. Excess STIM1 relative to Orai1 was previously suggested to be important for channel function. In pilot studies, we observed that a STIM1/Orai1 ratio of 3:1 was optimum for observing thapsigargin (TG)-evoked clustering in VSMCs. VSMCs, 24 to 48 hours after transfection, were detached and transferred to 35-mm glass-bottomed dishes with fresh culture medium and allowed to spread for 24 hours. Cells were serum-starved for 2 to 5 hours before imaging. During imaging, the extracellular solution contained (mmol/L): 130 NaCl, 5 KCl, 8 D-glucose, 10 HEPES, 1.5 CaCl₂, and 1.2 MgCl₂, titrated to pH 7.4 or 6.4 with NaOH. In some cells, eYFP-STIM1 fluorescence appeared as intense and large static patches; in these cases, the endoplasmic reticulum (ER) was judged to be perturbed and the cells were not used for investigation. We imaged VSMCs expressing low to moderate levels of STIM1 and Orai1 in an effort to mimic the physiological expression. After baseline image acquisition in 120 μL

Non-standard Abbreviations and Acronyms

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<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
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<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
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<td>HA</td>
<td>hemagglutinin</td>
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<td>PDGF</td>
<td>platelet-derived growth factor</td>
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<td>SERCA</td>
<td>sarco-endoplasmic reticulum Ca²⁺ ATPase</td>
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<td>STIM1</td>
<td>stromal interaction molecule 1</td>
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<td>TG</td>
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of extracellular solution, 120 μL of the same solution containing twice the final concentration of agent (eg, PDGF) was applied, minimizing mechanical disturbance. VSMCs were visualized on an Olympus IX-70 inverted microscope using a ×100 UPLAN objective (NA 1.35) supported by a DeltaVision deconvolution system (Applied Precision LLC) with SoftWoRx image acquisition and analysis software. Images were captured on a Roper CoolSNAP HQ CCD camera at 0.5-second exposure every 10 seconds, and epifluorescence was recorded using filter sets for FITC/TRITC. Imagery was used after acquisition to process and prepare micrographs. To compensate fluorophore bleaching, images were enhanced by a normalization feature so the pixel range, at 0.4% maximum saturation, was equal to the maximum range for each frame. Recordings were made at room temperature (21±2°C) unless otherwise stated.

Only VSMCs containing eYFP-STIM1 and mCherry-Orai1 that clustered in response to TG or PDGF were included. Quantification of clustering used Image J particle analysis point picker in a region of interest of each cell at the optimal focal point. Positive identification was a spot of intense mCherry fluorescence with 0.4 to 3.0 μm lateral diameter. Such spots included all focal intensities of this size and so clusters were recognized as new spots arising in response to PDGF or TG. In Figure 3E, this can be seen as the difference between the basal number of focal intensities (Pre) and the number after exposure to PDGF or TG. Clustering as determined by mCherry analysis was confirmed qualitatively as coclustering by visual inspection of the eYFP fluorescence overlay on mCherry clusters (Figure 3H and Online Supplement). The data were multilevel, with repeated exposures per cell, which required statistical methods that account for this. Modeling the combined dataset indicated that data followed a Poisson distribution and so a multilevel Poisson model was used. Plots compare freely derived and hypothesis-driven estimated mean counts with associated 95% confidence intervals. The hypothesis tested was that PDGF does not elevate wild-type Orai1 mutants at pH 7.4 at 21°C and wild-type Orai1 at pH 6.4 at 21°C, though not for the wild-type Orai1 at pH 7.4 at 37°C. Further details and discussion of the statistical methods are provided in the Online Supplement.

Intracellular Ca²⁺ Measurement
VSMCs were incubated with 2 μmol/L fura-2AM for 1 hour at 37°C followed by a 0.5-hour wash at room temperature. Measurements were made at room temperature on a 96-well plate reader (FlexStation, Molecular Devices). The change (Δ) in intracellular calcium (Ca²⁺) concentration was indicated as the ratio of fura-2 emission intensities for 340 nm and 380 nm excitation (F ratio). The recording solution contained (mmol/L): 130 NaCl, 5 KCl, 8 D-glucose, 10 HEPES, and 1.2 MgCl₂, titrated to pH 7.4 with NaOH, with 0.3 mmol/L CaCl₂ or without (Ca²⁺ free). Recordings were made at room temperature. Data are presented as mean±SEM, where n is the number of independent experiments and N the number of individual wells used in the 96-well plates.

Immunostaining
VSMCs, 48 hours after transfection, were detached and transferred to glass cover slips with fresh culture medium and allowed to spread for 24 hours. After treatments, the cells were fixed in 2% paraformaldehyde for 5 minutes, and, if stated, permeabilized in 0.1% TritonX-100 for 10 minutes at room temperature. Transfected cells were either incubated with anti-HA 1:500 (Covance) or anti-GFP (Abcam) as stated and for 1 hour at room temperature, then washed and incubated with the appropriate secondary Dylight 594 IgG (Stratec Scientific, Jacksons Immuno Research). All cells were mounted onto glass slides using ProLong Gold antifade (Molecular Probes, Invitrogen, Paisley, United Kingdom).

Reagents
Synta 66 was synthesized by Dr R. Foster (Leeds). Other reagents were from Sigma unless indicated otherwise.

Results
Constitutive Subcellular Localization and Dynamics of Orai1 and STIM1
The VSMCs adhered to the substrate as thin structures such that there was difficulty distinguishing plasma membrane from intracellular membranes and other structures. To investigate Orai1 that spanned the plasma membrane, we generated human Orai1 containing extracellular HA epitope tag and intracellular GFP ([HA]-Orai1-GFP). Nonpermeabilized cells were exposed to anti-HA antibody to label only surface-exposed Orai1. The [HA]-Orai1-GFP was evident throughout VSMCs, but there was thin, more intense staining along the cell perimeter (Figure 1A). Anti-GFP antibody failed to label these cells because they were nonpermeabilized (Figure 1B). After cell permeabilization, anti-HA staining was similar but vesicular structures were now also evident (Figure 1C). Anti-GFP antibody, which was also detected by red secondary antibody, gave a similar staining pattern to anti-HA antibody (Figure 1D). The data suggest that Orai1 localized to the plasma membrane. This Orai1 was evident at the cell perimeter and across the surface of these flat cells. Visualization of plasma membrane Orai1 dynamics was therefore possible across the entire VSMC. The data suggest that the tags did not prevent surface localization of Orai1 and that Orai1 existed in intracellular vesicles as well as the plasma membrane.

For simultaneous live-cell imaging of Orai1 and STIM1 dynamics, we used human Orai tagged with mCherry to visualize it as a red protein and human STIM1 tagged with eYFP to visualize it as a green protein (Figure 1E and 1F). Care was taken to use VSMCs expressing the minimum levels of STIM1 and Orai1 required for detection, avoiding overexpressing cells. The appearance of mCherry-Orai1 was similar to that of [HA]-Orai1-GFP labeled by anti-HA antibody in permeabilized cells (Figure 1E; compare with Figure 1C). There was Orai1 throughout the cells but areas of greater intensity were observed in dynamic structures: intracellular vesicles of 0.4 to 1 μm diameter; plasma membrane ruffles; and plasma membrane spiny protrusions (Figure 1E). Membrane ruffles, which are actin-rich waves, rippled away from the cell edges (Online Video I). Spiny protrusions emanated from the ruffles, appearing and disappearing (Figure 1E [arrowhead] and Online Video I).

eYFP on STIM1 restricts STIM1 from the plasma membrane.¹⁷ Therefore, eYFP tagged STIM1 does not address plasma membrane STIM1,¹⁸ only STIM1 localized to the ER or other intracellular structures. In VSMCs, the eYFP-STIM1 mostly had localization that was distinct from mCherry-Orai1 (Figure 1F). Particularly obvious was separation of the proteins in peripheral regions, which contained mCherry-Orai1 but not other intracellular structures. In VSMCs, the eYFP-STIM1 was taken to use VSMCs expressing the minimum levels of STIM1 and Orai1 required for detection, avoiding overexpressing cells. The appearance of mCherry-Orai1 was similar to that of [HA]-Orai1-GFP labeled by anti-HA antibody in permeabilized cells (Figure 1E; compare with Figure 1C).

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The data suggest that under nonstimulated conditions, Orai1 and STIM1 were localized as expected; constitutively dynamic; and operating largely independently.
Redistribution and Coclustering of Orai1 and STIM1 in Response to TG

It is expected that Orai1 and STIM1 redistribute and cocluster in response to passive store depletion evoked by TG, an inhibitor of sarco-endoplasmic reticulum Ca\(^{2+}\) ATPase (SERCA). Consistent with this expectation, VSMCs exposed to TG showed redistribution of eYFP-STIM1 into clusters (Figure 2A and 2B). Similarly, mCherry-Orai1 redistributed into clusters that were distinct from the Orai1-containing intracellular vesicles (Figure 2C and 2D). eYFP-STIM1 and mCherry-Orai1 colocalized once the clusters formed (Figure 2E and 2F and Online Video III). The coclusters were static after they had formed and were evident except in regions where eYFP-STIM1 had not been initially expressed (Figure 2E and 2F and Online Video III). The data suggest that Orai1 and STIM1 dynamically redistributed and coclustered in VSMCs, as reported for other cell types exposed to TG.

Ca\(^{2+}\) Release But Not Redistribution or Clustering in Response to PDGF

To investigate the relevance of STIM1 and Orai1 dynamics to the action of PDGF, we exposed VSMCs to PDGF instead of TG. PDGF evokes Ca\(^{2+}\) release and then Orai1-dependent Ca\(^{2+}\) entry in human saphenous vein VSMCs. As expected, STIM1 also contributed substantially to PDGF-evoked Ca\(^{2+}\) entry in these cells (Online Figure I). This Orai1- and STIM1-dependent Ca\(^{2+}\) entry is blocked specifically by the Synta 66 compound (S66) without effect on Ca\(^{2+}\) release. S66-sensitive Ca\(^{2+}\) entry occurred within 180 seconds after PDGF exposure, and it was the dominant Ca\(^{2+}\) signal after 300 seconds (Online Figure II).

PDGF failed to induce redistribution or coclustering of mCherry-Orai1 and eYFP-STIM1 (Figure 3A through 3D and Online Video IV). mCherry-Orai1 continued in its constitutive pattern in vesicular structures, ruffles, and spiny protrusions (Figure 3C and 3D and Online Video IV). Even at a concentration of 500 ng/mL, PDGF failed to evoke changes...
in the localization or dynamics of mCherry-Orai1 (Online Figure III). To be sure that the PDGF-resistant eYFP-STIM1 and mCherry-Orai1 were capable of clustering, the same VSMCs were first exposed to PDGF and then TG (Figure 3E). There was no change in response to PDGF but marked reorganization and coclustering when TG was applied (Figure 3E). For quantification, intense spots of mCherry-Orai1 fluorescence were counted before PDGF, after PDGF, and then after TG (Figure 3E). PDGF had no effect on the number of Orai1 spots, but the number of spots was significantly increased by TG (Figure 3E; for the statistical testing of hypotheses, Online Supplement). We interpret the spots before PDGF (Figure 3E) as intracellular vesicles, or possibly small ruffles. Evaluation of 52 VSMCs showed that PDGF never caused redistribution of mCherry-Orai1 or coclustering of eYFP-STIM1 and mCherry-Orai1.

The above recordings were made at room temperature, but STIM1 is heat-sensitive. Therefore we repeated the experiments at 37°C (Figure 3F through 3I and Online Video V). At this higher temperature, there were also no effects of PDGF on mCherry-Orai1 dynamics and there was no coclustering with eYFP-STIM1 (Figure 3G and 3I) until TG was applied (Figure 3H and 3I and Online Video V). The effect of TG was more marked at 37°C (Figure 3I). We interpret the TG-induced spots as clusters because they colocalized with redistributed STIM1 (Figure 3H).

The data suggest that PDGF activates Orai1 channels in VSMCs without causing redistribution or coclustering of Orai1 and STIM1, contrasting with the effects of TG.
We hypothesized that Ca^{2+}/H{\text{+}} influx through the activated Orai1 channels was sufficient to keep the stores full, restoring the Ca^{2+}/H{\text{+}} that had been released by PDGF so that there was no store depletion and thus no trigger for redistribution or clustering. To test the hypothesis, we first inhibited Orai1 channels using S66 (Figure 4A through 4E). PDGF now promptly evoked mCherry-Orai1 redistribution and coclustering with eYFP-STIM1 (Figure 4C through 4E and Online Video VI).

To investigate the specific role of Orai1 with more certainty, we made the R91W mutation in mCherry-Orai1 (Figure 5A through 5E) because this mutation prevents Orai1 function without affecting its localization. In these experiments, it was striking that PDGF promptly evoked mCherry-Orai1-[R91W] redistribution and coclustering with eYFP-STIM1 (Figure 5D through 5E and Online Video VII). Statistical analysis is provided in Figure 5F and the Online Supplement. The E106A mutation was also made in mCherry-Orai1 because it is an alternative approach for inhibiting ion permeation. Significant clustering of mCherry-Orai1-[E106A] was evoked by PDGF (Figure 5G; compare with Figure 3E).

The data suggest that PDGF was capable of redistributing and coclustering mCherry-Orai1 and eYFP-STIM1 but that these events were normally prevented by Ca^{2+} entry through Orai1 channels. That is, wild-type mCherry-Orai1 channels must have been activated by PDGF and generated Ca^{2+} entry that maintained the stores replete, preventing store depletion that would have resulted in clustering of the ER Ca^{2+} sensor STIM1. The wild-type mCherry-Orai1 channels were, therefore, behaving physiologically and yet they were activated by PDGF without clustering.
PDGF-Evoked Redistribution and Clustering Conferred by Acidosis

The above data suggest that redistributions and coclustering of Orai1 and STIM1 were not involved in responses to PDGF. Nevertheless, the data also show that the redistribution and coclustering processes exist in VSMCs. Therefore, we hypothesized that there may be conditions in which these processes are required by VSMCs. Previous studies on other cell types have suggested that Orai1 channels are strongly inhibited by acidification of the extracellular medium to pH 6.0 to 6.5, potentially through the glutamate residue E106 in Orai1.22 Such acidification occurs in ischemia and conditions of stress or pathology.23,24 Therefore, we investigated VSMCs and their PDGF responses in acidic conditions.

We first investigated if acidic extracellular pH inhibited Ca\(^{2+}\) influx through Orai1 channels in VSMCs (Figure 6A). VSMCs were pretreated with TG and then extracellular Ca\(^{2+}\) was added back to observe Ca\(^{2+}\) entry (Figure 6A). Previous studies showed that this Ca\(^{2+}\) entry is strongly inhibited by S66 and suppressed by Orai1 siRNA in VSMCs.8 This Ca\(^{2+}\) entry was inhibited by 65.5% at pH 6.4 and the fitted Hill equation suggested 50% inhibition at pH 6.6 (Figure 6A). Therefore, moderate acidity significantly reduced S66-sensitive Ca\(^{2+}\) entry in VSMCs.

The eYFP-STIM1 and mCherry-Orai1 dynamics were observed in VSMCs at pH 6.4 and in response to PDGF at pH 6.4 (Figure 6B through 6F). Application of PDGF caused highly significant redistribution and coclustering (Figure 6D through 6F; Online Supplement and Online Video VIII).

The data suggest that redistribution and coclustering of Orai1 and STIM1 are important in VSMCs when Ca\(^{2+}\) flux through Orai1 channels is compromised by acidosis.

Discussion

Primary findings of this study are that (1) in nonstimulated conditions, Orai1 and STIM1 were dynamic and mostly independent of each other; (2) in nonstimulated conditions, Orai1 was localized to intracellular vesicles and fairly uniformly distributed in the plasma membrane, except for intensities in ruffles and spiny protrusions; (3) in nonstimulated conditions, eYFP-STIM1 was localized to the ER and associated with...
Figure 5. PDGF-evoked clustering of nonconducting Orai1 mutants. Live cell imaging for a VSMC cotransfected with eYFP-STIM1 (green) and mCherry-Orai1-[R91W] (red). The VSMC was exposed to 100 ng/mL PDGF. Example images are shown for 40 seconds before PDGF (A) and then 0, 100, 200, and 280 seconds after PDGF (B through E). The small image in the top left corner shows a wider view of the cell, where the white box indicates the region selected for the main panels. In the main panels of C and D, aspects of the VSMC are highlighted by double-lined arrows to indicate examples of STIM1-Orai1-[R91W] coclusters induced by PDGF. The scale bar is 5 μm. F, Quantification of results of the type illustrated (A through E). Shown are the freely derived (black circles) and hypothesis-driven (red bars) estimated mean counts with 95% confidence intervals. G, For the same type of experiment but in which the mutation in Orai1 was E106A. Spots of intense mCherry-Orai1-[mutant] fluorescence were counted before (Pre) and 240 to 300 seconds after PDGF or vehicle (Veh.) exposure, and then 100 to 240 seconds after TG. The numbers of experiments were F, n/N=3/5 (PDGF) and 3/10 (Veh.) for R91W; and G, n/N=3/7 (PDGF) and 3/6 (Veh.) for E106A.
dynamic comet-like structures; (4) PDGF activated physiologically functional (i.e., store-filling) Orai1 channels without causing redistribution or clustering of Orai1 and STIM1; (5) Ca\(^{2+}\)-influx through PDGF-activated Orai1 channels inhibited redistribution or coclustering of Orai1 and STIM1; and (6) inhibition of Orai1 channels by extracellular acidosis changed the PDGF response so that it became associated with redistribution and clustering of Orai1 and STIM1. The findings suggest that redistribution and coclustering of Orai1 and STIM1 are not normally required for PDGF-evoked activation of Orai1 channels. These nonclustered channels nevertheless serve to maintain Ca\(^{2+}\) in the stores, much as clustered Orai1 channels serve to refill depleted stores. Despite this finding of activated nonclustered Orai1 channels, the study does not suggest lack of importance of the redistribution and clustering phenomena. Instead, it suggests that they become important when there is risk of store depletion, for example, in situations in which Orai1 channels are compromised such as in acidosis. Online Figure IV provides a diagrammatic summary of the findings and possible interpretations.

TG depletes stores by blocking SERCA and allowing domination of constitutive Ca\(^{2+}\) leak. The depletion is detected by STIM1, which is an ER Ca\(^{2+}\) sensor whose

**Figure 6. PDGF-evoked clustering of wild-type Orai1 during acidosis.** A, Summary data for intracellular Ca\(^{2+}\) measurement from VSMCs (n=3/26). Cells were pretreated with 2.5 \(\mu\)mole/L TG in Ca\(^{2+}\)-free solution, washed in Ca\(^{2+}\)-free buffer (pH 6.4, 6.7, 7.1, or 7.4), and then extracellular Ca\(^{2+}\) (0.3 mmol/L) was added at pH 6.4, 6.7, 7.1, or 7.4. The maximum amplitude of the Ca\(^{2+}\) add-back signal was measured and fitted curve is a Hill equation. B through E, Live cell imaging for a VSMC cotransfected with eYFP-STIM1 (green) and mCherry-Orai1 (red). The VSMC was imaged at pH 6.4 and exposed to 100 ng/mL PDGF and then 2.5 \(\mu\)mol/L thapsigargin (TG). Example images are shown for 20 seconds before PDGF (B), 0 and 230 seconds after PDGF (C and D), and then 140 seconds after TG (E). The panels of D contain double-lined arrows that indicate examples of STIM1-Orai1 coclusters induced by PDGF at pH 6.4. The scale bar is 5 \(\mu\)m. F, Quantification for results of the type illustrated in B through E. Shown are the freely derived (black circles) and hypothesis-driven (red bars) estimated mean counts with 95% confidence intervals. Spots of intense mCherry-Orai1 fluorescence were counted before (Pre) and 240 to 300 seconds after PDGF or vehicle (Veh.) exposure, and then 80 to 260 seconds after TG (n=3/6).
response to store depletion is redistribution and clustering (ie, clustering is an indicator of store depletion). We know, therefore, that PDGF did not cause store depletion in VSMCs because there was no clustering of eYFP-STIM1. That is, PDGF was causing Ca\(^{2+}\) release but not store depletion. We know that there was PDGF-evoked Ca\(^{2+}\) release because we could routinely measure it in Ca\(^{2+}\) measurement experiments. Furthermore, use of a mCherry-Orai1 mutant that failed to conduct Ca\(^{2+}\) led to PDGF-evoked clustering. Therefore, wild-type mCherry-Orai1 channels must have been activated by PDGF and then conferred Ca\(^{2+}\) entry that prevented store depletion. That is, mCherry-Orai1 channels were not only activated by PDGF but also behaving physiologically to keep Ca\(^{2+}\) stores replete, yet clustering was not involved. It follows that the stores should have depleted when Ca\(^{2+}\) influx through the PDGF-activated Orai1 channels became compromised (eg, by chemical blockade, mutation, acidosis), which is what we observed. Another inhibitor of the channels may be hypoxia, as suggested by studies on airway smooth muscle.\(^{25}\) Similarly, from an experimental perspective, it is important to recognize that Ca\(^{2+}\) influx is also lost when cells are studied in the absence of extracellular Ca\(^{2+}\), as has been common in other studies of physiological agonists. Although use of Ca\(^{2+}\)-free solution facilitates the distinction of Ca\(^{2+}\) release and Ca\(^{2+}\) entry, it leads the physiological agonist to cause store depletion because the stores cannot refill after Ca\(^{2+}\) release. Addition of Ca\(^{2+}\) back to the extracellular medium then leads to observation of Ca\(^{2+}\) entry through clustered Orai1 channels, as in TG experiments. In support of the suggestion that physiological agonists (in the presence of extracellular Ca\(^{2+}\)) normally cause Ca\(^{2+}\) release without causing significant store depletion, simultaneous measurements of cytosolic and ER Ca\(^{2+}\) in a human umbilical vein endothelial cell line showed that histamine caused substantial Ca\(^{2+}\) release but only modest loss of total ER Ca\(^{2+}\) content.\(^{26}\)

If there is Orai1 channel activation without redistribution and coclustering, how does the activation occur? One possibility is that a small fraction of the STIM1 and Orai1 proteins is constitutively assembled in nonclustered units. Such units would be initially inactive until PDGF generated a signal for STIM1’s CAD domain to bind Orai1 and open the channel. Given the complexity of the superficial ER,\(^{27}\) it is difficult to rule out a local depletion event as the activation signal, although it would need to be tightly contained to avoid evoking clustering. Alternatively the signal could be a second messenger or phosphorylation step arising from the activated PDGF receptor. A recent study suggested preformed STIM1-Orai1 units that enable rapid responsiveness in skeletal muscle,\(^{28}\) but such complexes required a long form of STIM1 that is not expressed in human saphenous vein VSMCs.\(^{18}\)

The finding that nonclustered Orai1 channels are sufficient to maintain stores replete raises a question about why the cells would then need to trigger the more dramatic process of redistribution and coclustering when Orai1 channels are inhibited or ER stress is threatened by some other means (eg, SERCA inhibition). We suggest that it is to maximize the chance for interaction between all of the Orai1 and STIM1 proteins in a way that increases the possibility for efficient store refilling without raising cytosolic Ca\(^{2+}\), thus minimizing the risk of store deple-

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Disclosures

None.

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Novelty and Significance

**What Is Known?**

- Orai1 forms calcium channels that are activated by PDGF and facilitate vascular smooth muscle cell remodeling.
- Activation of Orai1 channels by store depletion follows subcellular redistribution of STIM1 and Orai1 into clusters.
- Extracellular acidosis inhibits Orai1 channels.

**What New Information Does This Article Contribute?**

- PDGF can activate Orai1 channels without redistribution and clustering of STIM1 and Orai1 in vascular smooth muscle cells.
- Nonclustered Orai1 channels allow Ca²⁺ entry that prevents store depletion and clustering of STIM1 and Orai1.
- Acidosis inhibits Orai1 channels in vascular smooth muscle cells and, in so doing, confers PDGF-evoked clustering of STIM1 and Orai1.

STIM1 and Orai1 proteins have been identified as components of calcium-selective channels that are activated after depletion of intracellular calcium stores. These proteins exist in separate subcellular compartments before cellular stimulation and undergo major redistribution and clolustering in response to store depletion. It has been suggested that only after this clolustering do these proteins interact leading to calcium entry through channels formed by Orai1. Our studies of human vascular smooth muscle cells reveal that activation of these channels by PDGF does not require redistribution and clolustering, suggesting that an alternative mechanism may involve a fraction of the Orai1 and STIM1 proteins being constitutively coassembled. Furthermore, we show that nonclustered channels enable calcium entry that actively maintains calcium stores and prevents redistribution of Orai1 and STIM1. We show that this situation leads to a striking phenomenon in acidosis, which suppresses the PDGF-activated Orai1 channels, removing their inhibitory effect on redistribution. Consequently, in acidosis, PDGF evokes marked redistribution and co-clustering of STIM1 and Orai1. The results suggest that physiological activation of Orai1 channels does not require a central dogma previously proposed for activation of these channels but that redistribution is important as a safety mechanism to mitigate cell death in ischemia and pathology states.
Platelet-Derived Growth Factor Maintains Stored Calcium Through a Nonclustering Orai1 Mechanism But Evokes Clustering If the Endoplasmic Reticulum Is Stressed by Store Depletion

Lynn McKeown, Nicholas K. Moss, Paul Turner, Jing Li, Nikki Heath, Dermot Burke, David O'Regan, Mark S. Gilthorpe, Karen E. Porter and David J. Beech

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SUPPLEMENTAL MATERIAL

PDGF maintains stored calcium through a non-clustering Orai1 mechanism but evokes clustering if the ER is stressed by store-depletion

Lynn McKeown1,2, Nicholas K Moss1,2, Paul Turner1,2, Jing Li1,2, Nikki Heath1,2, Dermot Burke4, David O’Regan4, Mark S Gilthorpe3, Karen E Porter1,3, David J Beech1,2,3*

1Multidisciplinary Cardiovascular Research Centre and 2Faculty of Biological Sciences and 3Faculty of Medicine & Health, University of Leeds, Leeds, LS2 9JT, UK. 4Leeds Teaching Hospitals, General Infirmary, Great George Street, Leeds, LS1 3EX.

*Author for correspondence: Prof David J Beech, Garstang Building, Mount Preston Street, Faculty of Biological Sciences, University of Leeds, Leeds, LS2 9JT, England, UK; d.j.beech@leeds.ac.uk; Tel +44 (0) 113 34 34323/4727; Fax +44 (0) 113 34 34228.

Supplemental Methods

For siRNA transfection, cells (0.5–2 × 10⁶) were centrifuged (100×g) for 10 min, resuspended in Basic Nucleofector solution (Amaxa GmbH, Lonza GmbH, Cologne, Germany), mixed with 1 µmole/L short interfering (si) RNA to STIM1 (Ambion Europe Ltd, Applied Biosystems, Warrington, UK; CAAUUCGGCAAAACUCUGCtg, GCAGAGUUUUGCCGAAUUGtt) and transferred into a cuvette for electroporation (Amaxa). The scrambled control siRNA was Silencer Negative Control #1. Cells were transferred from cuvettes to pre-warmed culture medium and incubated in a 5 % CO2 incubator at 37 °C. Culture medium was changed after 24 hr, and measurements were made after a further 48 hr.

Intracellular Ca²⁺ measurement was performed as described in the main manuscript except the recording solution contained 1.5 mmole/L CaCl₂.

Supplemental Image Analysis

We initially sought to analyse images by quantifying the number of eYFP-STIM1 and mCherry-Orai1 co-localised spots (i.e. ‘yellow’ spots from the overlap of the green eYFP signal and the red mCherry signal). However, visualization of ‘yellow’ depended on the balance of the green and red colours and subjective decisions about the yellowness of a spot. Furthermore, STIM1 (green) comet-like dynamics occurred constitutively and overlapped dynamically with the constitutive Orai1 (red), generating a false background detection of ‘clusters’. We found that the counting of Orai1-only (red) clusters enabled unbiased analysis. Qualitatively, nevertheless, we are observed that counting yellow spots gave the same overall conclusion as counting red spots.

Supplemental Statistical Analysis of Images

The outcome data comprise counts and are assumed to follow an underlying Poisson distribution. Although each experiment contains only a small number of outcomes, by modelling the entire dataset combined it became possible to investigate model residuals and confirm this
distributional assumption. The data are also multilevel in nature, with repeated exposures (Pre, PDGF/vehicle or TG) per cell, which requires statistical methods that account for this. A multilevel Poisson model was therefore preferred to examine the combined data.

The hypothesis (H1) tested was that PDGF has negligible impact in elevating Orai1 spots (compared to its vehicle) for wild type Orai1 at pH 7.4 at 21±2 °C, whereas PDGF elevates spots for Orai1 mutants at pH 7.4 at 21±2 °C and wild type Orai1 at pH 6.4 at 21±2 °C, though not for the wild type Orai1 at pH 7.4 at 37 °C. The reference model (MH0) includes a factor to identify each experimental group separately, i.e. accommodating differences in amino acid sequence of Orai1 (wild type, R91W, E109A), pH (7.4 vs. 6.4), temperature (21 °C vs. 37 °C), and exposure type (Pre, PDGF/vehicle, or TG). This model estimates the mean count of each experiment by pooling all available data, thereby improving statistical power by assuming that all experiments are homogeneous apart from fixed differences arising due to either in genetics, pH levels, temperatures, and exposure types, plus all possible interactions. There are no constraints; hence means are ‘freely’ estimated. A second model (MH1) included a binary for the constraint implied under H1 (1 for where counts are hypothesised to be elevated, 0 otherwise). Two additional terms were also considered to allow for differences in the outcome at different pH levels (1 for pH 6.4, 0 for pH 7.4) and different temperatures (1 for 37 °C, 0 for 21 °C). Both models determine 95 % confidence intervals (CIs) associated with each estimated mean and the inspection of model residuals confirmed distributional assumptions. Models were estimated using the lme4 library in the statistical software package R version 2.14.2 (http://cran.r-project.org/) with confidence intervals derived via the delta method in the msm library.

Results from both models are illustrated in Figures 3 (E, I), Figure 5 (F, G) and Figure 6 (F) and the assessment of the H1 is summarised in Online Table I below. In the Figures, black circles and associated 95 % CIs depict the freely estimated mean counts of Orai1 spots for each experiment (i.e. allowing for fixed differences in sequence, pH, temperatures, and exposure types); red bars with corresponding 95 % CIs depict the hypothesis constrained estimated mean counts whilst accommodating fixed differences in pH levels and temperatures including interactions with H1. It can be seen from the Figures that the hypothesis fits the data well (p<0.001) and there are modest, though insubstantial differences in the base counts for the different pH and temperature levels; there are however substantial differences in the impact of PDGF and TG with different pH and temperature levels.

Online Table I quantifies the estimated impact of H1 whilst considering the separate roles of pH and temperature. The ‘baseline’ case is a reference group that comprises all pre or vehicle exposures or the PDGF exposure for only the wild type at pH 7.4 (at either 21 °C or 37 °C); the estimated baseline mean number of Orai1 spots is 17.9 (95 % CI: 14.7, 21.7). The impact of PDGF with either mutation or reduction in pH is to elevate the number of Orai1 spots to 48.5 (95 % CI: 40.2, 58.6). The reduction in pH is associated with a modest rise in the baseline number of spots to 22.3 (95 % CI 15.7, 31.6), whilst the impact of PDGF in combination with reduced pH yields a large elevation in the number of spots to 80.1 (95 %CI: 57.2, 112.3). The change in temperature to 37 °C is associated with a modest reduction in the number of spots to 12.1 (95 % CI: 7.7, 19.0), whilst the impact of TG in combination of the temperature change yields a large elevation in the number of spots to 95.1 (95 % CI: 61.7, 146.4).
**Online Table I:** Estimated impact of H1

<table>
<thead>
<tr>
<th>Reference</th>
<th>Mean Count</th>
<th>95% CI</th>
<th>p-value†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>17.9</td>
<td>14.7, 21.7</td>
<td>-</td>
</tr>
<tr>
<td>H1</td>
<td>48.5</td>
<td>40.2, 58.6</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>pH 6.4</td>
<td>22.3</td>
<td>15.7, 31.6</td>
<td>0.202</td>
</tr>
<tr>
<td>H1, pH 6.4</td>
<td>80.1</td>
<td>57.2, 112.3</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>37 °C</td>
<td>12.1</td>
<td>7.7, 19.0</td>
<td>0.068</td>
</tr>
<tr>
<td>H1, 37 °C</td>
<td>95.1</td>
<td>61.7, 146.4</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

†The p-value is the contrast significance to the reference baseline

The statistical approach combined sufficient data to verify the assumption that outcomes followed an underlying Poisson distribution. Modelling the entire dataset maximizes statistical power and reduces the number of hypotheses evaluated. It would be inappropriate to contrast differences between Pre, PDGF/vehicle and TG within the same experiment without accommodating the fact that cells undergoing the repeated exposures are not independent. A multilevel Poisson model was thus warranted.

A model with the H1 constraint only was initially considered but as there were substantial differences in the impact of TG with either pH 6.4 or at 37 °C, the model was revised to accommodate overall fixed effects due to differences in pH or temperature. However, it should be noted that despite including terms to accommodate pH and temperature differences, along with their interaction with H1, it is only the interaction terms that are of importance (p<0.001). In other words, the model that ignores the independent baseline differences of pH or temperature whilst retaining H1 interactions with both yields no change to the conclusion regarding H1 (results not shown). The impact of either pH 6.4 (p=0.202) or 37 °C (p=0.068) on baseline counts should not therefore be regarded as substantive.

**Supplemental Discussion**

We have obtained antibody labeling of endogenous Orai1 and STIM1 proteins in these cells. Therefore we initially sought to achieve information about clustering using this antibody approach. However, there are various reasons why the approach did not provide useful insight: (i) Antibody labeling of endogenous proteins did not confer capability for real-time imaging in living cells and so changes (dynamics) could not be tracked as PDGF was applied. It also did not distinguish between proteins that were capable of clustering and those that were not (Figure 1). It can only compare different cells treated with PDGF or vehicle control. Using this approach we found it was impossible to identify induced clustering events unambiguously. (ii) Antibody labeling inherently confers punctate (cluster-like) effects because multiple secondary antibodies assemble together. Therefore, even in basal conditions, it appeared like there were clusters but we could not be sure that they were true clusters (i.e. because there is no dynamic information, which is an essential characteristic of the STIM1-Orai1 event). (iii) Chemical fixation of cells, as required in antibody labeling, inherently causes clustering.

Co-immunoprecipitation studies were considered, but they would not provide useful information about the hypothesis. We show that PDGF activates Orai1 even though there is no clustering. Therefore, because STIM1 is involved in this activation of Orai1, PDGF is presumed to induce coupling (i.e. binding) of STIM1 to Orai1. Therefore, if co-immunoprecipitation experiments indicate increased association of STIM1 with Orai1 after PDGF exposure we could not know if it
arose because of induced binding or induced clustering. Even if it arose in part because of induced clustering, such data would not inform us whether or not any Orai1 channels were activated without clustering.

Supplemental Figure Legends

Online Figure I. Contribution of endogenous STIM1 to PDGF-evoked Ca\(^{2+}\) entry. Mean data for paired intracellular Ca\(^{2+}\) measurements from VSMCs transfected with scrambled siRNA (sc.si) or STIM1 siRNA (STIM1 si) (n=3/34). Shown is the change (Δ) in Ca\(^{2+}\) 360 s after exposure to 100 ng/mL PDGF in the continuous presence of 1.5 mmole/L extracellular Ca\(^{2+}\).

Online Figure II. Confirmation of S66-sensitive PDGF-evoked Ca\(^{2+}\) entry. Intracellular Ca\(^{2+}\) measurements from VSMCs pre-treated with vehicle or 5 μmole/L Synta 66. Cells were studied in the presence of 1.5 mmole/L extracellular Ca\(^{2+}\) and exposed to 100 ng/mL PDGF as indicated (n/N=3/41).

Online Figure III. Lack of clustering in response to 500 ng/mL PDGF. Example of a VSMC expressing eYFP-STIM1 (green) and mCherry-Orai1 (red) before (-20 sec) and after (240 sec) addition of PDGF in medium containing 1.5 mmole/L extracellular Ca\(^{2+}\).

Online Figure IV. Illustrations of the proposed mechanisms in VSMCs. The same section of the plasma membrane (PM) and a compartment of superficial sarco-endoplasmic reticulum (SR/ER, "store") are shown diagrammatically in each panel. The system is shown under three conditions: (A) in normal physiological extracellular pH (7.4) and the presence of PDGF; (B) in acidic extracellular pH (6.4) and the presence of PDGF; and (C) in normal physiological extracellular pH (7.4) and the presence of thapsigargin. Panel (B) is also suggested to be relevant to normal physiological extracellular pH (7.4) when: VSMCs are in the presence of Synta 66 or absence of extracellular Ca\(^{2+}\); or Orai1 function in VSMCs is disrupted by mutation (R91W, E106A). The pre-existing Orai1-STIM1 complex activated by PDGF without redistribution of Orai1 or STIM1 is labeled as “Unit”. When Orai1 and STIM1 redistribute and co-cluster, these assembles are labeled as “Cluster”. When Orai1 and STIM1 are not forming Ca\(^{2+}\) entry channels, they are labeled as being “Reserve” or having “Other functions”.

Supplemental Videos

Online Video I. Live cell imaging of mCherry Orai1 (red) in non-stimulated VSMCs. Images were taken every 10 s.

Online Video II. Live cell imaging of eYFP-STIM1 (green) in non-stimulated VSMCs. Images were taken every 10 s.

Online Video III. Live cell imaging of thapsigargin-evoked co-clustering (yellow) of eYFP-STIM1 (green) and mCherry-Orai1 (red). Images were captured 60 s before, then every 10 s until 300 s after application of 2.5 μmole/L thapsigargin (TG).

Online Video IV. Live cell imaging for a VSMC co-transfected with eYFP-STIM1 (green) and mCherry-Orai1 (red). Images captured 60 s before, then every 10 s until 300 s after application of 100 ng/mL PDGF.
Online Video V. Live cell imaging at 37 °C for a VSMC co-transfected with eYFP-STIM1 (green) and mCherry-Orai1 (red). Images were captured 40 s before and 300 s after application of 100 ng/mL PDGF and then 300 s after addition of TG.

Online Video VI. Live cell imaging for a VSMC co-transfected with eYFP-STIM1 (green) and mCherry-Orai1 (red). The cell was pre-treated for 15 min with 5 μmole/L Synta 66 and images were captured 40 s before, then every 10 s until 280 s after application of 100 ng/mL PDGF in the presence of Synta 66.

Online Video VII. Live cell imaging of a VSMC co-transfected with eYFP-STIM1 (green) and mCherry-Orai1-[R91W] (red). Images were captured 40s before, and then every 10 s until 280 s after the application of 100 ng/mL PDGF.

Online Video VIII. Live cell imaging for a VSMC co-transfected with eYFP-STIM1 (green) and mCherry-Orai1 (red). The cell was imaged at extracellular pH 6.4 and exposed to 100 ng/mL PDGF and then 2.5 μmole/L thapsigargin (TG).

Supplemental References

Online Figure I

ΔCa^{2+} at 360 s (Δ F ratio)

sc.si  STIM1 si
Fig. 2. Time course changes in ΔCa\textsuperscript{2+} (Δ F ratio) after application of PDGF (0.4 nM). The graph shows the effect of Synta 66 on the ΔCa\textsuperscript{2+} (Δ F ratio) over time. The vehicle control is also compared. The x-axis represents time in seconds (0-300).
Online Figure III

500 ng/mL PDGF

-20 sec  240 sec

eYFP-STIM1

mCherry-Orai1

A  B

Scale: 5 μm
Online Figure IV