Essential role for STIM1/Orai1-mediated calcium influx in PDGF-induced smooth muscle migration

Jonathan M. Bisaillon,* Rajender K. Motiani,* José C. Gonzalez-Cobos,* Marie Potier,
Katharine E. Halligan, Wael F. Alzawahra, Margarida Barroso, Harold A. Singer, David Jourd’heul,
and Mohamed Trebak

Center for Cardiovascular Sciences, Albany Medical College, Albany, New York

Submitted 21 July 2009; accepted in final form 25 January 2010

Bisaillon JM, Motiani RK, Gonzalez-Cobos JC, Potier M, Halligan KE, Alzawahra WF, Barroso M, Singer HA, Jourd’heul D, Trebak M. Essential role for STIM1/Orai1-mediated calcium influx in PDGF-induced smooth muscle migration. Am J Physiol Cell Physiol 298: C993–C1005, 2010. First published January 27, 2010; doi:10.1152/ajpcell.00325.2009.—We recently demonstrated that thapsigargin-induced passive store depletion activates Ca2+ entry in vascular smooth muscle cells (VSMC) through stromal interaction molecule 1 (STIM1)/Orai1, independently of transient receptor potential canonical (TRPC) channels. However, under physiological stimulations, despite the ubiquitous depletion of inositol 1,4,5-trisphosphate-sensitive stores, many VSMC PLC-coupled agonists (e.g., vasopressin and endothelin) activate various store-independent Ca2+ entry channels. Platelet-derived growth factor (PDGF) is an important VSMC promigratory agonist with an established role in vascular disease. Nevertheless, the molecular identity of the Ca2+ channels activated by PDGF in VSMC remains unknown. Here we show that inhibitors of store-operated Ca2+ entry (Gd3+ and 2-aminoethoxydiphenyl borate at concentrations as low as 5 μM) prevent PDGF-mediated Ca2+ entry in cultured rat aortic VSMC. Protein knockdown of STIM1, Orai1, and PDGF receptor-β (PDGFRβ) impaired PDGF-mediated Ca2+ influx, whereas Orai2, Orai3, TRPC1, TRPC4, and TRPC6 knockdown had no effect. Scratch wound assay showed that knockdown of STIM1, Orai1, or PDGFRβ inhibited PDGF-mediated VSMC migration, but knockdown of STIM2, Orai2, and Orai3 was without effect. STIM1, Orai1, and PDGFRβ mRNA levels were upregulated in vivo in VSMC from balloon-injured rat carotid arteries compared with uninjured control vessels. Protein levels of STIM1 and Orai1 were also upregulated in medial and neointimal VSMC from injured carotid arteries compared with uninjured vessels, as assessed by immunofluorescence microscopy. These results establish that STIM1 and Orai1 are important components for PDGF-mediated Ca2+ entry and migration in VSMC and are upregulated in vivo during vascular injury and provide insights linking PDGF to STIM1/Orai1 during neointima formation.

VASCULAR SMOOTH MUSCLE CELLS (VSMC) are one of the major cell types in blood vessels that play a central role in controlling the vascular tone and maintaining the integrity of the vessel wall (19, 49). Stimulation of VSMC membrane receptors by vasoactive compounds (e.g., vasopressin and angiotensin II) and growth factors (e.g., platelet-derived growth factor (PDGF) and fibroblast growth factor) that typically couple to isofoms of PLC induces phosphatididylinositol 4,5-bisphosphate breakdown into two second messengers: inositol 1,4,5-trisphosphate (IP3) and diacylglycerol. IP3 causes Ca2+ release through IP3 receptors from the sarcoplasmic reticulum (SR) (9). Since the amount of Ca2+ in the internal stores is finite, Ca2+ entry across plasma membrane channels is typically activated to replenish the empty stores and maintain a sustained increase in cytoplasmic Ca2+ necessary for signaling downstream to the nucleus. One of the major routes for Ca2+ entry into cells is through store-operated Ca2+ (SOC) entry (SOCE) channels, which are activated by internal Ca2+ store depletion (36). Depletion of internal Ca2+ stores induces the endoplasmic reticulum (ER)/SR-resident Ca2+ store sensor stromal interaction molecule 1 (STIM1) to translocate to areas near the plasma membrane to signal the activation of SOC channels encoded by Orai1 proteins (13, 14, 17, 35). However, in addition to Ca2+-selective Orai1 (33, 34), diverse SOC conductances encoded by transient receptor potential (TRP) canonical (TRPC) proteins have been described in many VSMC types (2, 3, 42). We and others recently demonstrated the upregulation of STIM1/Orai1 proteins in vitro in synthetic cultured VSMC compared with quiescent contractile freshly isolated VSMC (8, 34). We also showed that passive store depletion by thapsigargin activates Ca2+ entry in VSMC through STIM1/Orai1, independently of Orai2 and Orai3, and that store depletion-activated Ca2+ release-activated Ca2+ current-like currents are present in cultured synthetic migratory VSMC encoded by STIM1/Orai1 (34). Baryshnikov and co-workers (6) subsequently confirmed that SOCE is mediated by Orai1, independently of Orai2 and Orai3, and provided evidence for a functional association between Orai1, the Na+/Ca2+ exchanger, and the plasma membrane-associated Ca2+-ATPase pump in proliferating smooth muscle cells. However, under physiological agonist, i.e., PDGF, stimulation, the exact Ca2+ entry route activated remains uncertain. Despite concomitant depletion of IP3-sensitive stores, ligation of many PLC-coupled receptors activates, instead, distinct Ca2+ entry channels that do not depend on the state of filling of the internal stores (11, 39). For instance, the store-independent arachidonic acid-activated Ca2+ entry pathway mediated by arachidonic acid-activated channels is activated in response to PLC-coupled receptor stimulation under certain agonist conditions (39, 40). Recently, arachidonic acid-activated channels were shown to depend on plasma membrane STIM1 and to be contributed by Orai1 and Orai3 proteins (27, 28). Furthermore, stimulation of α1-adrenergic receptors activates store-independent Ca2+ entry through the diacylglycerol-activated TRPC6 channel (22). Heteromultimeric TRPC6 and TRPC7 channels were...
shown to contribute to vasopressin-activated Ca\(^{2+}\) entry in A7r5 smooth muscle cells (26), and increased Ca\(^{2+}\) entry induced by endothelin-1, another PLC-coupled receptor vasoactive agonist, was attenuated by TRPC3 knockdown (50).

Expression of PDGF can occur in all resident cells of the artery and in inflammatory cells that infiltrate the artery during disease (37). PDGF and its receptors are expressed at low or undetectable levels in normal vessels, but their expression is increased during vascular injury and atherosclerosis (37). It is now clearly established that PDGF plays a prominent role in migration of VSMC into the neointima following acute vessel injury or in the presence of atherosclerotic lesions (37). PDGF mediates its effects through binding to its receptor with subsequent Ca\(^{2+}\) mobilization. However, the molecular identity of the Ca\(^{2+}\) influx channels activated by PDGF, in general, and in VSMC, in particular, remains unknown.

In this study, we investigated the contribution of several Ca\(^{2+}\) channel candidates, known to be activated downstream of PLC isoforms, to the molecular composition of PDGF-activated Ca\(^{2+}\) entry channels in VSMC. We employed protein knockdown using silencing RNA (siRNA) against PDGF receptor-\(\beta\) (PDGFR\(\beta\)), the Ca\(^{2+}\) sensor STIM1, all three Orai isoforms, and the three TRPC isoforms expressed in primary VSMC (TRPC1, TRPC4, and TRPC6). We show that PDGF activates Ca\(^{2+}\) entry across the plasma membrane through the STIM1-Orai1 pathway, independently of TRPC1, TRPC4, and TRPC6 and Orai2 and Orai3 proteins. We also show that STIM1 and Orai1 knockdown inhibits VSMC migration in response to PDGF in vitro and that STIM1 and Orai1 are upregulated in vivo in neointimal VSMC during vascular injury.

**METHODS**

**Reagents.** Gd\(^{3+}\) was purchased from Acros Organ Inc.; recombinant rat PDGF-BB from R & D Biosystems; and 2-aminoethoxydiphenyl borate (2-APB) from Calbiochem. All siRNA sequences were obtained from Dharmace Inc., and, along with specific primers for rat STIM, Orai, and TRPC, are described elsewhere (34). The following primers were used: GCCATGCCCAACCTGCTCA (forward) and TCGGGCCTGTGGAAGCC (reverse) for rat PDGFR\(\beta\) and GUA-GAAACUCUUGGUGUAA (siRNA1) and ACAUCAAUACCGG-GACAU (siRNA2) for the siRNA against rat PDGFRB. Anti-STIM1 was purchased from BD Biosciences, anti-\(\beta\)-actin NH2-terminal domain from Sigma, and anti-Orai1 (extracellular; catalog no. ACCO-060) from Alomone. Western blot and immunofluorescence data were generated using the anti-Orai1 against the extracellular domain (Alomone), and similar results were obtained using anti-Orai1 antibodies from Sigma (catalog no. O8264) and ProSci (catalog no. 4041). All other chemical products were obtained from Fisher Scientific unless specified otherwise.

**VSMC dispersion and culture.** Male rats were euthanized by suffocation in a CO\(_2\) chamber. Aortas were dissected out into ice-cold physiological saline solution. Fat tissues and endothelium were removed. The artery was cut into small pieces and digested with a papain solution for 20 min at 37°C and then with a mixture of collagenase II and collagenase for 15 min at 37°C. The digestion solution was removed, and the cells were washed and gently liberated with a fire-polished glass pipette and transferred to culture plates.
Isolated VSMC undergo phenotypic modulation in culture that is complete within 30 h (18). Isolated VSMC were maintained in culture (45% DMEM-45% Ham’s F-12–10% FBS supplemented with L-glutamine) at 37°C, 5% CO2, and 100% humidity, passaged (synthetic), and used within one to six passages in all experiments.

**RT-PCR and real-time PCR.** Detailed protocol and sequences of specific primers are described elsewhere (34). Expression of TRPC, STIM, or Orai was compared with expression of the housekeeping gene GAPDH and measured using the comparative threshold cycle method, as previously described (1, 34).

**Cell transfections.** Transfections in VSMC were carried out using Nucleofector II (Amaxa) according to the manufacturer’s instructions. As a marker of cell transfection, 0.5 μg of green fluorescent protein was cotransfected with siRNA for identification of successfully transfected cells during experiments. As a control, we used a scrambled nontargeting sequence, as reported previously (34). For Ca2+ imaging experiments, cells were transfected with 10 μg of the siRNA of choice per 1 × 10^6 cells, seeded to round glass coverslips (for imaging) or plates (for various assays), and used 72 h after transfection.

**Ca2+ measurements.** Ca2+ was measured as described previously (1, 45, 47). Briefly, coverslips with attached cells were mounted in a Teflon chamber and incubated at 37°C for 45 min in culture medium (DMEM with 10% FBS) containing 4 μM fura 2-AM (Molecular Probes, Eugene, OR). Cells were then washed and bathed in HEPES-buffered saline solution (in mM: 140 NaCl, 1.13 MgCl2, 4.7 KCl, 2 CaCl2, 10 d-glucose, and 10 HEPES, with pH adjusted to 7.4 with NaOH) for ≥10 min before Ca2+ was measured. For Ca2+ measurements, fluorescence images of several cells were recorded and analyzed with a digital fluorescence imaging system (InCyt Im2, Intracellular Imaging, Cincinnati, OH). Fura 2 fluorescence at an emission wavelength of 510 nm was induced by excitation of fura 2 alternately at 340 and 380 nm. The ratio of fluorescence at 340 nm to that at 380 nm was obtained on a pixel-by-pixel basis. All experiments were conducted at room temperature.

**Western blots.** Cells were lysed using RIPA lysis buffer [50 mM Tris-HCl (pH 8), 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, and 0.2 mM EDTA], and 20–50 μg of proteins in denaturing conditions were subjected to SDS-PAGE (7.5%) and then electrotransferred onto polyvinylidene difluoride membranes. After the blots were blocked with 5% nonfat dry milk (NFDM) dissolved in Tris-buffered saline containing 0.1% Tween 20 (TTBS) for 2 h at room temperature, they were washed three times with PBS containing 0.1% Tween 20, blocked with 5% NFDM, and incubated overnight with primary antibodies against the proteins of interest. After washing three times with PBS containing 0.1% Tween 20, the blots were incubated with secondary antibodies conjugated with horseradish peroxidase. After washing three times with PBS containing 0.1% Tween 20, the blots were developed with an ECL chemiluminescence detection system (Amersham Biosciences, Piscataway, NJ). The bands were quantified by scanning densitometry using a densitometer (Model GS 510, Molecular Dynamics). The data were expressed as the ratio of the protein of interest to the housekeeping protein actin. The results were compared by one-way analysis of variance (ANOVA) followed by Tukey’s multiple comparison test. Data are presented as means ± SE. *P < 0.05.

**Fig. 2.** A–C: quantitative RT-PCR assessment of mRNA levels of stromal interaction molecule 1 (STIM) and Orai, transient receptor potential canonical (TRPC) isoforms, and PDGF receptor-β (PDGFRβ). C after knockdown by transfection of 2 specific silent RNA (siRNA) sequences used independently or a scrambled siRNA sequence used as a control. After 72 h, mRNA was isolated from cells and reverse transcribed, and quantitative PCR was performed using specific primers. Sequences for siRNA and primers for STIM, TRPC, and Orai are provided elsewhere (34); sequences for primers and siRNA against PDGFRβ are provided in METHODS. Data are representative of 3 independent transfections performed in triplicates. *P < 0.05. D and E: Western blots showing STIM1 and Orai1 protein knockdown after siRNA transfection as previously reported (34). Data are representative of 4 independent experiments.
TTBS for 5 min each and probed overnight at 4°C with specific primary antibodies [anti-STIM1 (1:250 dilution), anti-Orai1 (Alomone; 1:1,000 dilution), and β-actin NH2-terminal (NT) domain (1:2,000 dilution)] in TTBS containing 2% NFDM. On the next day, membranes were washed (3 times for 5 min each) with TTBS and incubated for 45 min at room temperature with a horseradish peroxidase-conjugated anti-mouse antibody (1:10,000 dilution; Jackson) for STIM1 and actin or anti-rabbit IgG for Orai1 (1:20,000 dilution; Jackson) in TTBS containing 2% NFDM. Detection was performed using the enhanced chemiluminescence reagent (Amersham).

Migration assays. VSMC were transfected with scrambled control or target siRNA, seeded, and allowed to form a monolayer for 48 h. The dish was scraped with the tip of a 100-μl pipette, and the resulting wound was washed with PBS. Culture medium containing 0.4% FBS with or without 100 ng/ml PDGF was added to the cells, which were incubated at 37°C in 5% CO2. SMC showed very little or no migratory response in the absence of PDGF. At different times, bright-field images were captured at 10 magnification (Leica DM IRB microscope) and analyzed (Adobe Photoshop CS3), and the total number of pixels in empty spaces inside the wound were counted and normalized to the control (scrambled siRNA) to gauge changes associated with knockdown of specific proteins. Data are represented as percentage of migration relative to the scrambled siRNA control.

Balloon injury of rat carotid arteries. The use of rats for these experiments has been reviewed and approved by the Institutional Animal Care and Use Committee at the Albany Medical College Animal Resource Facility, which is licensed by the US Department of Agriculture and the Division of Laboratories and Research of the New York State Department of Public Health and is accredited by the American Association for the Accreditation of Laboratory Animal Care. Male Sprague-Dawley rats (350–400 g body wt; Taconic Farms, Germantown, NY) were anesthetized with xylazine (5 mg/kg) and ketamine (70 mg/kg) via intraperitoneal injection, and balloon angioplasty was carried out essentially as previously described (20). Briefly, an embolectomy was performed as follows: a 2-F Fogarty balloon was inserted through a small arteriotomy in the external carotid artery and passed into the common carotid artery. After balloon inflation at 2 atm pressure, the catheter was partially withdrawn and reintroduced three times. After recovery from operation and anesthesia, animals received a postoperative dose of the analgesic buprenorphine (Buprenex; 0.02 mg/kg sc). Sham-operated animals were subjected to a similar procedure but without balloon insertion.

Immunofluorescence. Rats were euthanized by asphyxiation with CO2, and injured or noninjured carotid arteries were harvested and embedded in optimal cutting temperature compound and frozen by submersion in liquid nitrogen. Tissue sections (7 μm) were cut using a cryostat (Leica CM3050), mounted onto glass slides, and stored at −80°C. The carotid sections were fixed with precooled acetone for 10 min at 4°C and rinsed with PBS. The sections were incubated in a PBS washing buffer containing 0.1% Triton X-100 for 10 min, in blocking buffer (1× PBS, 5% goat serum, 0.5% fish gelatin, and 0.1% Triton X-100) for 30 min and then with primary antibody at 4°C overnight. A mouse monoclonal anti-STIM1 primary antibody was diluted 1:25 (BD Biosciences), or three rabbit polyclonal anti-Orai1 antibodies [anti-Orai1 extracellular [catalog no. ACC-060(6), Alomone], anti-STIM1 (1:250 dilution), anti-Orai1 (Alomone; 1:1,000 dilution), and β-actin NH2-terminal (NT) domain (1:2,000 dilution)] in TTBS containing 2% NFDM. On the next day, membranes were washed (3 times for 5 min each) with TTBS and incubated for 45 min at room temperature with a horseradish peroxidase-conjugated anti-mouse antibody (1:10,000 dilution; Jackson) for STIM1 and actin or anti-rabbit IgG for Orai1 (1:20,000 dilution; Jackson) in TTBS containing 2% NFDM. Detection was performed using the enhanced chemiluminescence reagent (Amersham).

Fig. 3. Representative traces showing average of PDGF-activated Ca2+ entry from several cells on single coverslips from scrambled control- or targeted siRNA-transfected VSMC on the same day. Results obtained with targeted siRNA against STIM1 and Orai1 (A), Orai2 (B), Orai3 (C), TRPC1 (D), TRPC4 (E), and TRPC6 (F) are shown. PDGF was used at 50 ng/ml for siOrai2 and at 25 ng/ml for all other recordings. Data are representative of 3–6 independent experiments per targeted siRNA. PDGF was first added in the absence of extracellular Ca2+; arrows show exact times of Ca2+ restoration to the extracellular milieu for each trace.
Orai1 NT domain [catalog no. 4041(34), ProSci], and anti-Orai1 (catalog no. O8264, Sigma), used independently, were diluted 1:25, 1:10, and 1:25, respectively, in blocking buffer. All three Orai1 antibodies showed similar results: robust upregulation of Orai1 in injured vessel sections. The sections were rinsed with washing buffer and then incubated with anti-mouse or anti-rabbit secondary antibody for 1 h at room temperature.

For STIM1 staining, an FITC-coupled anti-mouse secondary antibody (Molecular Probes) was diluted 1:300 in blocking buffer. For Orai1 staining, an FITC-coupled anti-rabbit secondary antibody (Molecular Probes) was diluted 1:300 in blocking buffer. Control sections were stained with secondary antibodies alone. Finally, the sections were mounted with a mounting medium containing 4′,6-diamidino-2-phenylindole (DAPI). The sections were later visualized using a confocal microscope (LSM 510 META). Images were collected using a ×63 oil immersion lens and a zoom setting of 0.7 (low magnification) or 2 (high magnification). LSM software was used to drive the hardware and image acquisition. For images collected at low magnification, intensity levels can be visualized without modification; for images collected at high magnification, ImageJ and Photoshop were used to provide a higher level of contrast for better visualization of protein subcellular distribution. Vertical sections were collected at high magnification at 0.5-μm intervals, with pinhole settings at 1 Airy unit. ImageJ Volume Viewer plug-in was used to generate a vertical cross-sectional image using trilinear interpolation.

Fig. 4. A: PDGF-activated Ca²⁺ entry after VSMC transfection with scrambled or targeted siRNA (against STIM1, Orai1, Orai2, Orai3, TRPC1, TRPC4, and TRPC6) was measured using fura 2, and extent of Ca²⁺ entry was analyzed in several cells originating from several independent experiments and is represented as the difference between the fura 2 signal before and after Ca²⁺ (2 mM) restoration. Results are averages of cells from 3–6 independent experiments per condition. Numbers within bars represent total number of cells for each condition. B: representative traces depicting effect of STIM1 and Orai1 simultaneous silencing on PDGF-mediated Ca²⁺ entry (n = 15). Condition with control scrambled siRNA-transfected cells (n = 22) is also shown. C: extent of Ca²⁺ entry in many scrambled control- and siOrai1 siSTIM1-transfected cells. Results are averages from 3 independent experiments; numbers within bars represent total number of cells. D: representative traces depicting effect of Orai1 (n = 15), siOrai1 + siOrai2 (n = 18), and siOrai1 + siOrai3 (n = 14) knockdown on PDGF-mediated Ca²⁺ entry compared with control scrambled siRNA-transfected cells (n = 20). E and F: Ca²⁺ release and entry (n = 16); control trace represents an average of 25 scrambled siRNA-transfected cells. G and H: Ca²⁺ release and entry in scrambled control- and PDGFRβ-transfected cells. Results are averages from 4 independent experiments; numbers within bars represent total number of cells. In all experiments, PDGF was used at a concentration of 50 ng/ml. *P < 0.05 vs. control for A, C, F, H, and I.
For immunofluorescence in cultured synthetic VSMC, cells were transfected with nontargeting siRNA or Orai1 siRNA (see above) and allowed to grow for 72 h on coverslips coated with collagen. At 4°C, cells were washed with PBS and fixed with paraformaldehyde. Cells were then permeabilized for 5 min with PBS-0.2% Triton X-100 and incubated in blocking buffer (PBS, 0.1% Triton X-100, and 0.5% fish gelatin) for 30 min. The cells were allowed to incubate overnight in anti-Orai1 primary antibody diluted in blocking buffer at 1:25 (Alomone) at 4°C. The cells were rinsed and then treated with anti-rabbit secondary antibody diluted in blocking buffer at 1:300 for 1 h. The coverslips were mounted in mounting medium containing DAPI and imaged using a wide-field microscope (Zeiss Axio Observer Z1) at low (∼/H11003 40) magnification. Images show intensity levels without modification. Nonpermeabilized cells were treated for immunofluorescence using the same protocol without Triton X-100. After treatment with secondary antibody, the cells were allowed to incubate in wheat germ agglutinin-Alexa Fluor 594 at 4°C for 10 min at 5 μg/ml. The coverslips were mounted with a mounting medium containing DAPI and imaged using a confocal microscope (LSM 510 META) under high magnification and processed for visualization as described above.

Statistical analysis. Data are expressed as the number of cells from several transfections (means ± SE). Each condition reflects results from several coverslips originating from at least two independent transfections. For statistical analysis, one-way ANOVA was carried out using Origin software. Differences were considered significant when P < 0.05.

RESULTS

Pharmacological characterization of PDGF-mediated Ca\(^{2+}\) entry. We recently showed that SOCE in human umbilical vein and human pulmonary artery endothelial cells and rat aortic VSMC is mediated by STIM1 and Orai1 independently of other Orai isoforms and TRPC proteins (1, 34). To test whether PDGF mediates Ca\(^{2+}\) entry in VSMC via store-dependent or store-independent channels, we used pharmacological inhibitors of SOCE and found that PDGF activates a Ca\(^{2+}\) entry pathway that displays classical SOCE features similar to those observed in nonexcitable cells, such as HEK-293 and rat basophilic leukemia cells. In primary cultured VSMC (synthetic), PDGF-evoked Ca\(^{2+}\) entry was essentially blocked with 5 μM Gd\(^{3+}\) and 30 μM 2-APB and, as recently demonstrated in HEK-293 cells (41), by 50 μM ML-9 (Fig. 1A), suggesting that STIM1 and Orai1 might be the molecular players in the PDGF-activated Ca\(^{2+}\) entry pathway in VSMC. We showed recently that SOCE in primary cultured VSMC displays a unique feature, i.e., concentrations of 2-APB as low as 5 μM are inhibitory (34). In additional experiments in cells incubated with Gd\(^{3+}\) (5 μM) or 2-APB (5 μM) before restoration of extracellular Ca\(^{2+}\), PDGF-activated Ca\(^{2+}\) entry was also sensitive to these concentrations of Gd\(^{3+}\) and 2-APB in a manner that is reminiscent of thapsigargin-activated SOCE in VSMC (34) (Fig. 1B). Results similar to those described in Fig. 1, A and B, were obtained from experiments with the VSMC cell line A7r5 (data not shown). Dose-response analysis of Gd\(^{3+}\) inhibition showed that Gd\(^{3+}\) concentrations as low as 0.5 μM substantially inhibited PDGF-activated Ca\(^{2+}\) entry (64.64 ± 3.98% inhibition, n = 19), whereas 5 μM Gd\(^{3+}\) essentially inhibited PDGF-activated Ca\(^{2+}\) entry (88.18 ± 2.61% inhibition, n = 19; Fig. 1C) in a manner reminiscent of that seen with thapsigargin-activated SOCE in HEK-293 cells (44).

Fig. 5. A: bright-field views of scratch wound migration assay in serum-free medium containing 100 ng/ml PDGF at 0–24 h after the wound in primary cultured VSMC transfected with scrambled control siRNA or siRNA against STIM1 or Orai1. B and C: data from 3 independent experiments (with 4 wells per condition) at 12 and 24 h after the wound. *P < 0.05.
**STIM1 and Orai1 mediate PDGF-activated Ca\(^{2+}\) entry in VSMC.** To determine the molecular identity of the plasma membrane Ca\(^{2+}\) channels activated by PDGF in primary cultured VSMC, we used siRNA against STIM1, Orai, and TRPC isoforms. Knockdown of STIM1, Orai, or TRPC isoforms in VSMC was achieved with two different siRNA sequences, used individually (see Ref. 34 for siRNA sequences). As measured by quantitative RT-PCR, siRNA sequences induced a marked decrease in their target mRNA levels 72 h after transfection (Fig. 2, A and B): by 82.14 \(\pm\) 1.34\% for siOrai1-1, 57.38 \(\pm\) 4.68\% for siOrai2-1, 65.67 \(\pm\) 3.55\% for siOrai3-1, 90.18 \(\pm\) 3.57\% for siSTIM1-1, 68.79 \(\pm\) 6.23\% for siSTIM2-1, 87.6 \(\pm\) 1.6\% for siTRPC1-1, 90.82 \(\pm\) 2.48\% for siTRPC4-1, and 66.1 \(\pm\) 8.24\% for siTRPC6-1 (\(n = 6\)). As assessed by Western blotting and reported previously (34), extensive characterization of the same siRNA in VSMC by our group showed that they induce a decrease in protein levels of their respective targets: by 74.7 \(\pm\) 7.5\% for STIM1-1, 58.3 \(\pm\) 7.0\% for Orai1-1, 58.8 \(\pm\) 6.4\% for Orai3-1, 61.8 \(\pm\) 7.7\% for TRPC1-1, 66.2 \(\pm\) 12.1\% for TRPC4-1, and 85.4 \(\pm\) 5.8\% for TRPC6-1 (\(n = 3\)). Representative Western blot analysis of STIM1 and Orai1 from cells transfected with scrambled control siRNA, siRNA against STIM1, or siRNA against Orai1 are shown in Fig. 2, D and E. Interestingly, downregulation of STIM1 or Orai1 using siRNA significantly suppressed PDGF-activated Ca\(^{2+}\) entry in primary cultured VSMC, whereas neither Orai2, Orai3, TRPC1, TRPC4, nor TRPC6 knockdown affected the amplitude of PDGF-induced Ca\(^{2+}\) entry in primary cultured VSMC (Fig. 3). Representative traces in Fig. 5 show an average of several VSMC originating from single coverslips 72 h after transfection with control scrambled siRNA or target siRNA and assayed on the same day. Knockdown of individual STIM, Orai, or TRPC isoforms in VSMC had no significant effect on SR Ca\(^{2+}\) release (data not shown). Figure 4A shows results from statistical analysis of Ca\(^{2+}\) entry measured using fura 2 imaging after knockdown of different proteins in several individual cells obtained from different coverslips from at least three independent transfections. The inhibition of Ca\(^{2+}\) entry compared with control was 53.58 \(\pm\) 8.36\% for siSTIM1 (\(n = 49\)) and 54.17 \(\pm\) 4.16\% (\(n = 50\)) for siOrai1, with no significant inhibition detected on knockdown of other proteins (Fig. 4A). Results are representative of two independent siRNA sequences per target gene and are normalized to control cells transfected with a scrambled siRNA (for sequence see Ref. 34). Simultaneous knockdown of STIM1 and Orai1 had an effect (55.43 \(\pm\) 5.08\% inhibition, \(n = 106\)) similar to knockdown of STIM1 or Orai1 alone, strongly arguing that both proteins are involved in the same pathway. Figure 4B shows a representative trace with average cells from the same coverslip, and Fig. 4C shows results from statistical analysis of several cells from

Fig. 6. A: bright-field views of scratch wound migration assay in serum-free medium containing 100 ng/ml PDGF at 0–24 h after the wound in primary cultured VSMC transfected with scrambled control siRNA or siRNA against STIM2, Orai2, or Orai3. B and C: data from 3 independent experiments (with 4 wells per condition) at 12 and 24 h after the wound.
three independent experiments. Given the incomplete knockdown of Orai2 and Orai3, the contribution of these proteins to SOCE in VSMC might be smaller. In a separate set of experiments, we compared double knockdown of Orai1 + Orai2 or Orai1 + Orai3 with single knockdown of Orai1. Figure 4D shows that the extent of SOCE inhibition is similar whether Orai1 is knocked down alone or in combination with Orai2 and Orai3. This lack of additivity suggests that Orai2 and Orai3 do not contribute significantly to SOCE in VSMC. However, we cannot rule out completely a small contribution from Orai2 and/or Orai3 to SOCE in VSMC. Figure 4, E and F, shows results from statistical analysis of three to four independent experiments where Ca^{2+} release and Ca^{2+} entry were measured in cells transfected with control siRNA and siRNA against Orai isoforms.

Protein knockdown of the PDGFRβ using two independent siRNA caused a substantial decrease in Ca^{2+} release and Ca^{2+} entry in response to PDGF. Figure 4G shows representative traces from individual coverslips, and Fig. 4, H and I, shows results from statistical analysis of Ca^{2+} release (82.24 ± 11.8% inhibition, n = 88) and Ca^{2+} entry (89.29% ± 6.87 inhibition, n = 88), respectively, from four independent experiments. The ability of siRNAs against the PDGFRβ to induce a substantial decrease in PDGFRβ mRNA was determined by quantitative PCR (Fig. 2C): 80.04 ± 1.12%
decrease for siPDGFRβ1 and 63.02% ± 3.3% decrease for siPDGFRβ2.

**STIM1, Orai1, and PDGFRβ are important for PDGF-induced VSMC migration.** Primary cultured VSMC transfected with scrambled control siRNA or siRNA against different STIM and Orai isoforms were used in a scratch wound assay to assess VSMC migration in response to PDGF stimulation. The cultured rat aortic VSMC used in our laboratory have a doubling time of 22–24 h. To avoid contributions from VSMC proliferation, wound closures were analyzed 12 h after the scratch, when VSMC migration is apparent and proliferation is minimal. siRNA against STIM1 or Orai1 led to a substantial decrease in the wound surface covered by VSMC in response to 100 ng/ml PDGF at 12 and 24 h after the wound: 46.4 ± 10.7% at 12 h for STIM1 and 30.4 ± 6.9% at 12 h for Orai1 (Fig. 5). However, STIM2, Orai2, and Orai3 silencing had no significant effect on VSMC migration (Fig. 6). Figure 7 shows that knockdown of PDGFRβ in VSMC using siRNA caused a substantial decrease in the wound surface covered by VSMC in response to 100 ng/ml PDGF at 12 and 24 h after the scratch compared with scrambled control-transfected cells: 48.31 ± 6.56% at 12 h vs. 60.46 ± 4.78% at 24 h (Fig. 7).

Recent studies showed upregulation of STIM1 and Orai1 proteins in proliferative migratory rat mesenteric arteries compared with quiescent freshly isolated VSMC from the same vascular bed (8), and our group recently reported similar findings in rat aortic VSMC (34). The involvement of STIM1 and Orai1 in PDGF-mediated VSMC migration in vitro prompted us to determine whether STIM and Orai proteins and PDGFRβ are upregulated in proliferative and migratory VSMC in vivo in an animal model of vascular disease.

**STIM1, Orai1, and PDGFRβ are upregulated in VSMC during vascular injury.** Although the role of Orai1 in neointima formation remains unknown, two recent reports described an essential role for STIM1 in neointima formation following arterial balloon injury in the rat (5, 16). Therefore, we sought to analyze the potential upregulation of STIM1, Orai1, and

![Fig. 8.](http://ajpcell.physiology.org/)

**Fig. 8.** A and B: hematoxylin-eosin-stained sections of carotid arteries from a time-matched (14 days) noninjured sham control rat and a balloon-injured rat (14 days after injury). M, media; NI, neointima; L, lumen. C: quantitative RT-PCR assessment of mRNA levels of STIM and Orai isoforms and PDGFRβ. mRNAs were isolated from VSMC from sham control and injured rat carotid arteries. Values are expressed as fold increase in mRNA in injured carotid arteries compared with sham control. Data were obtained from 3 control and 3 injured rats that were analyzed in 3–7 independent runs. D: Western blots showing higher levels of STIM1 and Orai1 protein expression in synthetic (cultured) VSMC than in quiescent freshly isolated VSMC, as demonstrated previously (34). Rat basophilic leukemia (RBL) cells were used in parallel as a positive control.
PDGFβ in VSMC in vivo in a rat model of vascular injury. We subjected carotid arteries of male rats to balloon injury (see Methods) and used sham-operated animals as controls. Our vascular injury model has been well described in numerous published studies over many years (7, 25). In contrast to diet-induced injuries, mechanical injuries are characterized predominantly by VSMC proliferation and migration, with only early and transient lymphocyte and monocyte infiltration and very few detectable immune cells days after the injury (43). Figure 8, A and B, shows representative hematoxylin-eosin-stained sections from sham control or injured carotid vessels 14 days after treatment. Figure 8C shows that mRNA levels encoding STIM1, Orai1, and PDGFβ are significantly upregulated in injured carotid arteries 14 days after injury compared with noninjured time-matched sham-operated rats: 12.03 ± 3.33 fold for STIM1, 15.13 ± 3.02 fold for Orai1, and 6.33 ± 0.44 fold for PDGFβ. However, the mRNA levels of STIM2, Orai2, and Orai3 were unaffected. As reported earlier (34), representative Western blots in Fig. 8D show that STIM1 and Orai1 upregulation also occurs in vitro in synthetic cultured VSMC, suggesting that contributions from infiltrating leukocytes to the increase in STIM1 and Orai1 in vivo are unlikely.

Although we showed in Fig. 2E that Orai1 antibody recognized a single band at the appropriate molecular weight that was decreased 72 h after transfection with specific siRNA against Orai1, strongly arguing for the specificity of this antibody for Orai1, we performed additional experiments in cultured VSMC to further validate the use of Orai1 antibody in immunofluorescence studies in vessel sections. We used a similar Orai1 knockdown approach followed by immunofluorescence staining with anti-Orai1 in cultured synthetic VSMC, as depicted in Fig. 9A. In these experiments, we used standard protocols with Triton X-100 permeabilization and visualization of images using a wide-field microscope under low-magnification conditions (see Methods) to process VSMC for immunofluorescence staining. Figure 9A clearly shows a dramatic decrease in Orai1 staining 72 h after treatment with siRNA against Orai1. To address the putative plasma membrane association of Orai1 in VSMC cells, we used immunofluorescence staining under nonpermeabilized conditions and then imaged the cells using a confocal microscope at high magnification. As shown in Fig. 9B, Orai1 staining colocalizes with that of wheat germ agglutinin, a well-known plasma membrane marker in nonpermeabilized cells, suggesting a plasma membrane localization of Orai1. Next, we used immunofluorescence staining to probe for STIM1 and Orai1 protein expression in vessel sections of carotid arteries from sham-operated and injured rats. Figure 10 shows increased protein expression of STIM1 and Orai1 in medial and neointimal VSMC from injured rat carotid arteries (14 days after injury) compared with medial VSMC from noninjured vessels. In Fig. 10, fluorescence images were obtained under similar conditions at low magnification to provide an accurate representation of differences in STIM1 and Orai1 fluorescence levels between the sham-operated and injured carotid vessels. To further characterize the subcellular distribution of Orai1 in tissues from control and injured carotid arteries, we collected vertical sections at 0.5-μm intervals using confocal microscopy (1.63 lens and zoom factor of 2). ImageJ Volume Viewer plug-in was used to generate a vertical cross section of the tissue slice, which shows a peripheral localization of Orai1, consistent with a plasma membrane association (Fig. 11). Orai1 staining appeared to be more polarized in cells from sections of injured carotid arteries than from sections of control vessels; additional studies are clearly needed to understand the contribution of this polarized Orai1 staining to VSMC migration in vitro and in vivo.

Fig. 9. A: VSMC were transfected with non-targeting siRNA (siControl) or siRNA targeting Orai1 (siOrai1), fixed, and processed for immunofluorescence under permeabilized conditions using anti-Orai1; 4’6’-diamidino-2-phenylindole (DAPI) was used as a nuclear marker. Images are representative of 3 independent experiments. A reduction in Orai1 expression is clearly detected in siOrai1 images. B: VSMC were fixed and processed for immunofluorescence under nonpermeabilized conditions (without Triton X-100) using anti-Orai1 (Orai1), wheat germ agglutinin (WGA)-Alexa Fluor 594 was used as a plasma membrane marker and DAPI as a nuclear marker, and images were collected using confocal microscopy. Arrows indicate colocalization between Orai1 and WGA at the plasma membrane (Merge, yellow staining). Images were modified in Adobe Photoshop to a higher level of contrast for better visualization.
DISCUSSION

Agonist binding to G protein-coupled receptors or receptor tyrosine kinase activates isoforms of PLC to cause Ca\(^{2+}\)/H\(\text{1001}\) release from the SR and Ca\(^{2+}\)/H\(\text{11001}\) entry from the extracellular space. Receptor-activated Ca\(^{2+}\) entry channels are of two major types: 1) SOC channels activated by the fall of the Ca\(^{2+}\) concentration within the SR through the action of IP\(_3\) on its receptor and 2) store-independent channels activated by diverse mechanisms involving, among others, second messengers (31). Identification of the Ca\(^{2+}\)-binding protein STIM1 has provided insights into the molecular identity of SOC channels and the signaling mechanisms connecting internal Ca\(^{2+}\) store depletion to this Ca\(^{2+}\) entry (for review see Ref. 35). STIM1, the long-sought Ca\(^{2+}\) sensor in the ER/SR (24, 38) that is capable of oligomerization and reorganization into punctuate structures in defined ER-plasma membrane junctional areas, upon store depletion, signals the activation of the SOC channel at the plasma membrane composed of the mem-

![Image](https://example.com/image.png)

**Fig. 10.** Immunofluorescence staining of carotid artery sections from rats subjected to balloon injury (C and F; 14 days after injury) and control noninjured vessels from sham-operated animals (A, B, D, and E) using anti-Orai1 (B and C) and anti-STIM1 (E and F) antibodies followed by a secondary antibody coupled to FITC. A and D: secondary antibody-alone control. Brackets indicate neointima (NI) in injured vessels. Data are representative of results obtained independently from sections originating from 3 control and 3 injured rats. M, media; L, lumen.

![Image](https://example.com/image.png)

**Fig. 11.** Carotid artery sections from rats subjected to balloon injury (B; 14 days after injury) and control noninjured vessels from sham-operated animals (A) were processed for immunofluorescence staining using anti-Orai1; DAPI was used as a nuclear marker. Vertical sections were collected at 0.5-\(\mu\)m intervals using confocal microscopy at high magnification. A representative image is shown at right. ImageJ Volume Viewer plug-in was used to generate a vertical cross section (yellow line) of the tissue slice. Images were modified in Adobe Photoshop to a higher level of contrast for better visualization. Orai1 shows a peripheral subcellular localization, which is consistent with a plasma membrane association.
brane protein Orai1 (15, 48). The mechanisms of STIM1-Orai1 coupling are beginning to emerge and appear to involve direct binding of a minimal, highly conserved ~107-amino acid region of STIM1 to the NH2 and COOH termini of Orai1 to open the Ca2+ release-activated Ca2+ channel (23, 29, 32, 51). However, a wide variety of store-dependent and store-independent nonselective cation conductances activated downstream of PLC-coupled receptors have been described by different groups in many cell types, including VSMC (3, 21, 31).

Molecular candidates for these cation channels include homomultimers and heteromultimers within ORAI and TRP family members, as well as heteromultimers of ORAI/TRP isoforms (4, 12, 46).

In vitro and in vivo studies in mouse mutants have established PDGF as a major proproliferative and promigratory growth factor for many cell types of mesenchymal origin, including fibroblasts and smooth muscle cells (10). The importance of PDGF signaling during cardiovascular diseases such as restenosis and atherosclerosis is clearly established, whereby PDGF attracts PDGFRβ-expressing medial VSMC, thus promoting their accumulation in the neointima (37). PDGF receptors are receptor tyrosine kinases that couple to PLCγ activation and cause an increase in cytoplasmic Ca2+ via plasma membrane Ca2+ entry channels. However, the molecular identity of PDGF-activated Ca2+ entry channels in VSMC remains unknown. In this report we show that, in cultured synthetic VSMC, PDGF activates a Ca2+ entry pathway pharmacologically reminiscent of classical SOCE, namely, inhibition by low concentrations of lanthanides (5 μM Dy3+), 2-APB (5 μM), and ML-9 (50 μM). We also show that PDGF-induced Ca2+ entry was substantially inhibited by knockdown of Orai1, STIM1, and PDGFRβ, whereas knockdown of Orai2, Orai3, TRPC1, TRPC4, and TRPC6 had no significant effect, establishing STIM1/Orai1 as the Ca2+ entry route activated in response to PDGFRβ stimulation in VSMC.

The primary cultured VSMC used in this study (also known as synthetic) are dedifferentiated VSMC that have lost their contractile markers and are highly proliferative and migratory compared with the quiescent freshly isolated VSMC (19, 30). These synthetic VSMC recapitulate most of the phenotype and gene expression patterns of proliferative and migratory VSMC found in vivo during vascular diseases such as restenosis and atherosclerosis (19, 30). We and others previously showed increased STIM1 and Orai1 protein expression and upregulated SOCE in these cultured VSMC compared with their quiescent freshly isolated counterparts (8, 34). Here we show that knockdown of STIM1 or Orai1 inhibits VSMC migration in vitro in response to PDGF, whereas STIM2, Orai2, and Orai3 silencing had no significant effect, suggesting that the STIM1-Orai1 pathway is the mediator of PDGF action on PDGFRβ in VSMC. Furthermore, we also show that, during vascular injury, medial and neointimal VSMC upregulate the mRNA and protein expressions of PDGFRβ, STIM1, and Orai1. Two recent studies demonstrated that in vivo silencing of STIM1 with use of viral particles encoding STIM1-targeted short hairpin RNA in rat balloon-injured vessels inhibits neointima formation (5, 16). However, Orai1 upregulation or its contribution to VSMC migratory phenotype in vivo has not been studied. Our results powerfully complement these studies by providing a plausible mechanism for the role of STIM1/Orai1 in neointima formation. Clearly, additional studies are needed to understand the roles of all STIM and Orai isoforms in mediating Ca2+ entry in response to various growth factors/agonists, so we might gain insights into the use of these proteins as targets for vascular disease therapy.

ACKNOWLEDGMENTS

We are very grateful to Dr. David W. Saffen (Fudan University, Shanghai, China) for generous gifts of reagents and advice. We also gratefully acknowledge the administrative support of Wendy Vienneau and Jo Anne Evans. Present address of M. Potier: INSERM U921 Nutrition Croissance Cancer, Faculté de Médecine, Bat Dutrochet, 10 Bd Tonnellé, 37032 Tours, France (e-mail: marie.potier-cartereau@univ-tours.fr).

GRANTS

This work was supported by National Institute of Environmental Health Sciences Early Career Grant K22 ES-014729 and supplements (K22 ES-014729SI and K22 ES-014729S2) allocated through the American Recovery and Reinvestment Act (to M. Trebak).

DISCLOSURES

No conflicts of interest are declared by the author(s).

REFERENCES


---

**C1005**

STIM1/ORAI AND PDGF-MEDIATED SMOOTH MUSCLE MIGRATION

---

Downloaded from http://ajpcell.physiology.org/ by 10.220.33.6 on April 1, 2017