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Store-Independent Orai1/3 Channels Activated by Intracrine LeukotrieneC_4: Role in Neointimal Hyperplasia

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Abstract

Rationale—Through largely unknown mechanisms, Ca^{2+} signaling plays important roles in vascular smooth muscle cell (VSMC) remodeling. Orai1-encoded store-operated Ca^{2+} entry (SOCE) has recently emerged as an important player in VSMC remodeling. However, the role of the exclusively mammalian Orai3 protein in native VSMC Ca^{2+} entry pathways, its upregulation during VSMC remodeling and its contribution to neointima formation remain unknown.

Objective—The goal of this study was to determine the agonist-evoked Ca^{2+} entry pathway contributed by Orai3; Orai3 potential upregulation and role during neointima formation after balloon-injury of rat carotid arteries.

Methods and Results—Ca^{2+} imaging and patch clamp recordings showed that while the platelet-derived growth factor (PDGF) activates the canonical Ca^{2+} release-activated Ca^{2+} (CRAC) channels *via* store depletion in VSMC, the pathophysiological agonist thrombin activates a distinct Ca^{2+}-selective channel contributed by Orai1, Orai3 and STIM1 in the same cells. Unexpectedly, Ca^{2+} store depletion is not required for activation of Orai1/3 channel by thrombin. Rather, the signal for Orai1/3 channel activation is cytosolic leukotrieneC_4 produced downstream thrombin receptor stimulation through the catalytic activity of leukotrieneC_4 synthase. Importantly, Orai3 is upregulated in an animal model of VSMC neointimal remodeling and in vivo Orai3 knockdown inhibits neointima formation.

Conclusions—These results demonstrate that distinct native Ca^{2+}-selective Orai channels are activated by different agonists/pathways and uncover a mechanism whereby leukotrieneC_4 acts through *hitherto* unknown intracrine mode to elicit store-independent Ca^{2+} signaling that promotes

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DISCLOSURES
None.

vascular occlusive disease. Orai3 and Orai3-containing channels provide novel targets for control of VSMC remodeling during vascular injury or disease.

Keywords

Calcium signaling; Orai3; Orai1; STIM1; vascular smooth muscle; neointima formation; ion channel

INTRODUCTION

Ca^{2+} is a universal signal that controls a variety of cell functions of major importance in health and disease. Stimulation of specific phospholipase C (PLC)-coupled receptors generates spatio-temporal Ca^{2+} signals pivotal for control of cellular responses such as gene transcription, contraction, secretion, migration, proliferation and apoptosis[1, 2]. These receptor-activated Ca^{2+} entry pathways comprise: i) store-operated Ca^{2+} entry (SOCE) channels activated by inositol 1,4,5-trisphosphate (IP_3)-mediated depletion of Ca^{2+} from the endoplasmic reticulum (ER) stores[3] and ii) store-independent Ca^{2+} entry (non-SOCE) channels activated by largely unknown mechanisms that do not depend on the state of filling of ER stores[4]. Whether store-operated and store-independent Ca^{2+} channels are concomitantly activated by the same agonist or whether specific agonists selectively activate specific Ca^{2+} channels remain unknown.

SOCE channels mediate the highly Ca^{2+}-selective, Ca^{2+} release-activated Ca^{2+} (CRAC) current[5]. The past several years have brought about remarkable advances in our understanding of the SOCE pathway with the identification of STIM1 as the ER Ca^{2+} sensor[6, 7] and Orai1 as the pore-forming unit of CRAC channels at the plasma membrane (PM)[8–10]. Depletion of ER Ca^{2+} stores causes oligomerization of STIM1 and its accumulation into punctuate structures within regions of close contacts (10–25 nm) between the ER and PM[11]. Direct STIM1-Orai1 interaction involving the binding of a minimal C-terminal 100 amino acid domain of STIM1 (called SOAR/CAD/OASF/CCb, spanning the coil-coiled region) to the C-and N-termini of Orai1, activates local Ca^{2+} entry through CRAC channels[12–15]. CRAC currents were originally measured in cell lines such as RBL mast cells and Jurkat T cells; In RBL cells for example, CRAC current density is 1–2 pA/pF at −100mV[5]. The paucity of electrophysiological recordings of native store-operated Ca^{2+}-selective conductances in response to physiological agonists in primary cell types such as vascular smooth muscle cells (VSMCs) is largely due to the tiny single-channel conductance of CRAC channels (~15femto-Siemens) [2] coupled to the low expression levels of STIM1 and Orai1 proteins in these primary cells; CRAC current density is 0.1–0.3 pA/pF at −100mV in cultured VSMCs[16].

While it is generally accepted that Orai1 homologs, Orai2 and Orai3 mediate SOCE and CRAC currents when co-expressed with STIM1 in HEK293 cells, a very interesting finding from a large number of mammalian cell types and tissues studied so far is that native SOCE is exclusively mediated by Orai1, despite concomitant and abundant expression of Orai2 and Orai3 proteins in these systems[17]; the exception is an instance where Orai3 encodes SOCE in breast cancer cell lines that expresses estrogen receptors[18, 19]. This raises the interesting possibility that homo- and hetero-multimers contributed by Orai2 and Orai3 might encode Ca^{2+} selective channels distinct from CRAC that are activated by alternative store-independent mechanisms that would enhance the diversity of cellular Ca^{2+}-selective conductances. In fact, while a great deal of attention has focused on the SOCE pathway, there is increasing evidence for a number of non-SOCE pathways in various cell types[4]. The likely signal for activation of store-independent Ca^{2+} entry pathways are second messengers generated downstream PLC activity. However, i) the molecular identity, ii) the exact

mechanisms of second messenger action, iii) the specific agonist requirement, iv) the ionic currents mediating these pathways, and v) the physiological and pathophysiological functions controlled by these pathways remain largely obscure. One exception is the store-independent Ca^{2+} selective channel mediating the Arachidonic Acid (AA)-regulated Ca^{2+} (ARC) current[20]. ARC channels have been shown to be activated by AA in HEK293 cells and to be contributed by both Orai1 and Orai3 subunits[21] and regulated by a specific pool of PM-resident STIM[22]. Members of the transient receptor potential canonical (TRPC) family are either activated by diacylglycerol (DAG) produced downstream of PLC (TRPC3/6/7) [23] or by store depletion through STIM1 (TRPC1/3/4/5/6)[24]. However, TRPCs are non-selective cation channels carrying mainly Na^+ ions and have protein structures that are different from Orai channels, suggesting that TRPCs and Orais contributions to native Ca^{2+} entry pathways and cell functions are likely non redundant.

VSMCs are one of the major cell types in blood vessels and play major roles in vessel integrity and control of blood pressure[25]. Unlike cardiac and skeletal muscles that are terminally differentiated, VSMCs are plastic in nature and retain the ability to switch in vivo from a contractile excitable phenotype to a proliferative migratory non-excitable phenotype (also called synthetic[25]), a condition that can be recapitulated in culture. This VSMC phenotype modulation is essential for vascular development, angiogenesis and repair. However, its dysfunction contributes to vascular diseases such as atherosclerosis, hypertension, restenosis and leiomyosarcomas. VSMC phenotypic modulation is characterized by downregulation of L-type Ca^{2+} channels and upregulation of STIM1 and Orai1[16, 25, 26]. STIM1 and Orai1 were shown to be required for VSMC phenotypic switch in vitro and neointima formation in animal models of vascular injury[16, 26–30], supporting a role for agonist-activated Ca^{2+} entry pathways in driving VSMC phenotypic modulation during disease. Indeed, mitogenic migratory and inflammatory agonists such as the platelet-derived growth factor (PDGF) and thrombin are major contributors to vascular remodeling that are heavily produced and see the expression of their receptors increased during vascular injury[31–33].

In this study, we apply improved low noise high resistance (>16GΩ) whole-cell patch clamp recordings, amplify and measure reliably tiny highly Ca^{2+} selective SOCE and non-SOCE currents from primary synthetic VSMCs in response to PDGF and thrombin. We show that PDGF and thrombin, two established VSMC pathophysiological agonists, activate distinct Ca^{2+}-selective channels involving distinct mechanisms; while PDGF activates classical store-dependent CRAC currents mediated by Orai1, thrombin activates a store-independent current encoded by both Orai1 and Orai3 that requires intracellular leukotrieneC_4 (LTC_4) produced downstream receptor activation. Finally, VSMC Orai3 and LTC_4-activated currents are upregulated in vivo in an animal model of carotid vessel injury and Orai3 knockdown using specific shRNA-encoding lentiviruses inhibits these currents as well as VSMC remodeling and neointima formation.

METHODS

List of reagents used throughout the study methods and detailed experimental procedures on VSMC dispersion and culture, cell transfections, Ca^{2+} measurements, patch clamp electrophysiology, FRET microscopy and balloon injury of rat carotid arteries are provided in the Online Supplement.

RESULTS

Thrombin-activated Ca^{2+} entry and currents are distinct from SOCE and CRAC currents

Previous studies from our group showed that the pro-proliferative agonist PDGF activates Ca^{2+} entry through classical SOCE in rat synthetic VSMCs[30]. Interestingly, Fura2 Ca^{2+} imaging protocols with agonist stimulation in a nominally Ca^{2+}-free solution followed by restoration of Ca^{2+} (2mM) to the extracellular milieu revealed that thrombin (at maximal concentrations, 100nM) activates a Ca^{2+} entry pathway in primary synthetic rat aortic VSMCs that is additive to the Ca^{2+} entry activated by PDGF (100ng/ml; Figure 1a), suggesting that these two Ca^{2+} entry pathways are distinct. Whole-cell patch clamp recordings using a pipette solution containing 20 mM of the chelator BAPTA (to cause maximal store depletion) demonstrated the activation of an inwardly rectifying Ca^{2+}-selective CRAC current (sampled at −100mV; Figure 1b). Subsequent addition of thrombin (100nM) to the same cells consistently activated an additional current (Figure 1b), suggesting that thrombin mediates Ca^{2+} entry through a CRAC-independent pathway. CRAC and thrombin-activated currents were recorded in Ca^{2+}-containing (20mM) bath solutions and amplified in divalent-free (DVF; Na^+ is the charge carrier) solutions. Both these currents have reversal potentials of around +60mV (Online Table I). Figure 1c shows the current/voltage (I/V) relationships of CRAC and thrombin-activated currents and their additivity. Positions on traces where I/V curves for currents are taken in this figure and all subsequent figures are indicated by the color-coded signs (e.g. asterisks). Statistics on whole-cell current densities measured in DVF solutions upon store depletion (1^{st}) and subsequent addition of thrombin (2^{nd}) are shown in Figure 1d. Statistical analyses of patch clamp data (mean ± range; n) and p values for comparisons done from this figure and all subsequent figures are reported in Table 1.

Thrombin-activated Ca^{2+} entry and currents are store-independent

Since thrombin-activated Ca^{2+} entry and membrane currents are additive to SOCE and CRAC currents activated by either passive store depletion or physiologically by PDGF, two major questions arise: 1) does thrombin, by virtue of activating PLCβ and producing IP_3, cause store-depletion? And 2) is store depletion required for activation of Ca^{2+} entry and currents in response to thrombin? Surprisingly, using three different approaches, detailed below, we found that i) thrombin does not cause sustained store depletion, only reversible Ca^{2+} release and ii) the Ca^{2+} entry and currents it activates do not require store depletion:

1. Ca^{2+} imaging experiments with sequential addition of PDGF followed by thrombin and *vice versa* at maximally saturating concentrations (500nM thrombin and 500ng/ml PDGF) in nominally Ca^{2+} free solutions showed that while PDGF caused store depletion, thrombin did not. Using the following protocol: “PDGF was added first in nominally Ca^{2+} free followed by extracellular Ca^{2+} restoration for 6min (to prevent excessive Ca^{2+} leak from ER) in the continuous presence of PDGF (to maintain IP_3 production), followed by thrombin addition in nominally Ca^{2+} free”, thrombin failed to cause Ca^{2+} release. However, when thrombin was added first followed by PDGF using the same protocol, PDGF, though added second, caused comparable Ca^{2+} release to when it was added first (Online Figure I a–d).

2. The use of the ER-targeted Ca^{2+} dye Cameleon D1ER coupled to Förster resonance energy transfer (FRET) fluorescence microscopy showed that while thapsigargin was very effective at causing ER Ca^{2+} store depletion, maximal concentrations of thrombin had only a small and transient effect (Figure 1e). Similar experiments showed that PDGF also caused store depletion but at a faster rate and to a lesser extent than thapsigargin (Online Figure Ie).

3. Whole-cell patch clamp recordings using a pipette solution where free Ca^{2+} was buffered to 150nM with BAPTA showed that PDGF activated a Ca^{2+}-selective current reminiscent of CRAC that showed the typical depotentiation in DVF solutions[34] (Figure 1f). Inclusion of heparin in the patch pipette, destined to inhibit IP_3 receptors, completely abrogated CRAC currents activated by PDGF as would be expected for a store-dependent current (Figure 1g). However, thrombin-activated currents did not depotentiate in DVF solutions and were normally activated in the presence of heparin (Figure 1i). The I/V curves taken where indicated in traces by color-coded asterisks are represented in Figure 1j and statistics for PDGF and thrombin are shown in Figure 1h and 1k respectively. Statistical analyses of patch clamp data from each figure (mean ± range; n) and p values for group comparisons to control are reported in Table 1.

Thrombin activates a Ca^{2+}-selective entry pathway mediated by STIM1, Orai1 and Orai3

To determine the molecular identity of thrombin-activated Ca^{2+} entry pathway, we used an unbiased molecular knockdown approach targeting all Orai and TRPC isoforms expressed in synthetic VSMCs. We used the following: i) infection with specific short hairpin (shRNA)-encoding lentiviruses; ii) transfection with specific small interference RNA (siRNA) sequences (Online Table III) and iii) transfection with dominant negative (DN) Orai constructs (Figure 2d, image 1) showed that thrombin-mediated Ca^{2+} entry requires STIM1, Orai1 and Orai3 but was independent of Orai2 and the three TRPC1/4/6 isoforms found expressed in rat synthetic VSMCs[16] (Figure 2b, c, d). Knockdown of STIM1, Orai1 and Orai3 is shown in Figure 2a while knockdown of Orai2 is documented in Online Figure IIa. Statistical analyses on the extent of Ca^{2+} entry is shown in Figure 2d. Please note throughout that the representative Ca^{2+} imaging traces represent averages from several cells on the same coverslip as indicated by n. For statistical analysis, the numbers between parentheses next to each column of bar graphs (x, y) represent: x= number of independent runs and y=total number of cells from all these runs. All p values for comparisons are listed in Online Table II. The unexpected involvement of Orai3 in this pathway prompted us to use yet an additional siRNA sequence against Orai3 and demonstrate that Orai3 protein knockdown inhibits thrombin-activated Ca^{2+} entry without effecting Orai1 and STIM1 protein expression (Online Figure II b–d).

Thrombin-activated Ca^{2+} entry and currents require cytosolic LTC_4

Since store depletion is not required for thrombin-activated Ca^{2+} entry and currents, we systematically evaluated the role of second messengers produced downstream thrombin receptor in the activation of this pathway. IP_3 dialysis through the patch pipette exclusively activated CRAC currents recognized by their pharmacology and their depotentiation in DVF solutions and addition of a DAG analog (1-oleoyl-2-acetyl-sn-glycerol; OAG) to the bath solution activated non-selective currents mediated by TRPC6 in VSMCs (not shown). Given the molecular similarity between the thrombin-activated Ca^{2+} entry and ARC channels (dependence on STIM1, Orai1 and Orai3), we applied exogenous arachidonic acid (AA; 8μM) to VSMCs which consistently activated a Ca^{2+} selective current that did not depotentiate in DVF solutions and was not additive to currents activated by thrombin (Figure 2e–g). The addition of thrombin first followed by AA confirmed this lack of additivity (Online Figure IIe–g).

The use of pharmacological reagents targeting the downstream AA metabolism pathway ruled out the requirement of cyclooxygenases (COX1/2) and leukotrieneA_4 hydrolase but suggested requirement of leukotrieneC_4 synthase (LTC_4S) in thrombin-mediated Ca^{2+} entry (not shown). Therefore, we tested whether introduction of LTC_4 directly into the cytosol through the patch pipette could activate a current reminiscent of thrombin and AA-activated

currents. We used a concentration of LTC_4 (100nM) shown to be physiologically relevant[35]. LTC_4 dialyzed through the patch pipette activated a Ca^{2+}-selective current in VSMCs under Ca^{2+}-containing and DVF solutions and this current had a reversal potential of +60mV (Online Table I), did not depotentiate in DVF solutions and more importantly was not additive to thrombin-activated currents (Figure 3a–c). However, a closer leukotriene, LTB_4 failed to activate whole-cell currents when dialyzed through the patch pipette, while subsequent addition of thrombin to the same cells consistently activated Ca^{2+} selective currents (Online Figure IIIa–c). Furthermore, addition of LTC_4 to the bath solution did not activate any current, suggesting that LTC_4 acts from the inside and not *via* its specific PM G-protein-coupled receptors (GPCRs; Online Figure IIId, e). Stimulation of VSMCs with thrombin lead to an increase of LTC_4 production after either 5 or 15 min in presence of thrombin as measured using competitive ELISA (Figure 3d; p values for comparisons are provided in Online Table II). Molecular knockdown of LTC_4S with either siRNA transfection (with 2 independent siRNA sequences) or shRNA infection (encoding a third sequence) reduced LTC_4S protein expression (Figure 3e) and inhibited thrombin-activated Ca^{2+} entry (Figure 3f, g) and membrane currents (Online Figure IIIh-k for siRNA and S3l-o for shRNA). Control experiments showed that SOCE activated by thapsigargin was insensitive to LTC_4S knockdown (Online Figure IIIf, g). Significantly, currents activated by direct introduction of LTC_4 into cells were insensitive to LTC_4S protein knockdown (Figure 3h–k).

LTC_4-activated currents require STIM1, Orai1 and Orai3

Knockdown experiments using shRNA-encoding lentiviruses showed that STIM1 was required for current activation by direct application of LTC_4 in the patch pipette (Figure 4a–d), arguing that STIM1 is downstream of LTC_4 action. Control experiments demonstrating that STIM1 knockdown also abrogates CRAC currents in the same cells are shown (Online Figure IV). We showed that Orai1 and Orai3 are both required for thrombin-activated Ca^{2+} entry (Figure 2). Here we show that LTC_4 delivered through the patch pipette into VSMCs also requires both Orai1 (Figure 5a–d) and Orai3 (Figure 5i–l) as demonstrated with knockdown using shRNA-encoding lentiviruses; these results further strengthen the idea that LTC_4 and thrombin activate the same Ca^{2+} entry pathway in VSMC. Control experiments showed that, in the same cells, store-dependent CRAC currents require Orai1 (Online Figure Va–d) but not Orai3 (Online Figure Ve–h). Infection with shRNA-encoding lentiviruses against Orai3 (shOrai3) caused downregulation of Orai3 protein levels with no effect on its closest homologue, Orai1 (Figure 5e–h), thus establishing the specificity of shOrai3 for subsequent in vivo studies.

Orai3 and LRC currents are upregulated in medial and neointima VSMC after vascular injury

Cultured synthetic VSMCs reminiscent of vascular occlusive disease used so far in this study have upregulated protein levels of Orai3 by comparison with quiescent freshly isolated VSMCs that are reminiscent of healthy vessels (Online Figure VIa; please note that β-actin is also known to be upregulated in proliferative VSMCs in vitro and in vivo). Given the established importance of thrombin in vascular injury and vascular occlusive disease[31, 33, 36] and the specific involvement of Orai3 in the thrombin-activated Ca^{2+} entry pathway, we tested whether Orai3 is also upregulated in vivo in a model of vascular remodeling and neointima formation after balloon injury of rat carotids. The validated shOrai3 lentiviruses along with lentiviruses encoding non targeting control shRNA (shNT) were used in vivo in this injury model to determine whether preventing Orai3 upregulation after injury could have inhibitory effects on vascular remodeling and neointima formation. ShOrai3 achieved significant Orai3 knockdown in cultured VSMCs (Figure 5g, h) and infection with shOrai3

and shNT lentiviruses led to essentially 100% infection of VSMCs in vitro as visualized using green fluorescent protein (GFP) encoded by the lentiviruses (Online Figure VIb).

Injury of rat left carotid arteries lead to the apparition of LTC_4-regulated Ca^{2+} (LRC) currents in medial and neointimal VSMC acutely isolated from injured arteries; medial VSMC from non-injured arteries showed no significant LRC currents upon dialysis of LTC_4 into the cells (Figure 6a–c). The I/V relationships of whole-cell LRC currents from all these different types of cells are shown in Figure 6d and summary of data is shown in Figure 6e; statistical analyses of data (mean ± range; n) are depicted in Table 1. Similarly, we found that CRAC currents, activated by store depletion with dialysis of 20mM BAPTA through the patch pipette, were evident in medial and neointimal VSMC acutely isolated from injured carotid arteries, while medial VSMC from non-injured vessels showed no detectable CRAC currents (Online Figure VII). These results are consistent with previous data from our lab reporting upregulation of Orai1 and SOCE upon vascular injury.[16, 29, 30]

Injury of left rat carotid arteries also caused a significant upregulation of Orai3 proteins in lysates of medial and neointimal VSMCs at day 14 post-injury (Figure 6f) as well as that of Orai1 proteins as previously shown[29]. Efficient vessel infection by shOrai3 or shNT-encoding lentiviruses was documented by the expression of lentivirus-encoded GFP (driven by a separate promoter) in medial and neointimal VSMCs from injured and infected left carotids 14 days post injury (Figure 6f).

Orai3 is required for LRC currents and neointima formation in vivo

Survival surgery involving transduction of injured carotid arteries of anesthetized animals with shOrai3 lentiviruses caused a significant attenuation of Orai3 protein upregulation at day 14 post injury with no significant effect on Orai1 expression (Figure 6f–h) by comparison to control shNT. This *in vivo* Orai3 knockdown corresponded functionally to a decrease in whole-cell LRC current densities in medial (by ~55%) and neointimal VSMC (by ~48%) from injured carotids 14 day post-injury (Figure 7a–d). The I/V relationships of whole-cell LRC currents from different experimental conditions are shown in Figure 7e, f and summary of data is shown in Figure 7g; statistical analyses of data (mean ± range; n) are shown in Table 1.

Upon vascular injury, increased in neointimal thickening was evident in injured vessels 14 days post-injury compared to normal control vessel from sham-operated rats (Figure 7h). Importantly, Orai3 *in vivo* knockdown caused a significant reduction in neointima size compared to shNT control as observed on Hematoxylin-eosin sections (Figure 7h). Both the neointimal size (N) and the ratio of neointima/media (N/M) were reduced upon Orai3 knockdown compared to control (Figure 7i, j).

DISCUSSION

Our data reveal a store-independent mechanism of Orai channel activation *via* PLC-coupled receptors and support a model whereby Orai1/3 channel activation by thrombin requires cytosolic LTC_4 produced upon thrombin receptor ligation. We show that these LTC_4-Regulated Ca^{2+} (LRC) channels do not require store depletion for activation. We also show that unlike PDGF, thrombin does not cause sustained store depletion. The fact that thrombin does not deplete stores despite the presence of a Ca^{2+} spike in nominally Ca^{2+} free solutions is likely due to transient IP_3 production since the protease-activated receptor 1 (PAR1), which is the major thrombin receptor in VSMC is used once and then discarded. However, In other cell types such as fibroblasts and endothelial cells, signaling by thrombin is maintained by delivery of new PAR1 receptors to the plasma membrane from a preformed intracellular pool[37]. Interestingly, previous data in cultured endothelial cells showed that

thrombin activates SOCE and CRAC currents that are dependent on store depletion and classical STIM1/Orai1 pathway[38]. The implications of all these results are that not only do different agonists activate distinct Ca^{2+} entry pathways in a given cell type but the same agonist could activate a different Ca^{2+} entry route depending on the cell type in question. Our work introduces a novel paradigm whereby specific PLC-coupled agonists activate specific Ca^{2+}-selective entry pathways in the same cells. This implies that PM receptors ensure the specificity of the Ca^{2+}-selective channel activated and likely the downstream Ca^{2+}-responsive transcription factors and corresponding physiological functions. This diversity of Ca^{2+}-selective conductances through Orai subunit heteromultimerization and alternative activation mechanisms would presumably help increase the repertoire of spatial cellular Ca^{2+} microdomains for the purpose of selective Ca^{2+} signaling in complex mammalian organisms.

Thrombin has been shown to have multiple pleiotropic effects: it impacts on VSMC contractility and proliferation and is a major contributor to vascular remodeling. Thrombin is produced massively after vascular injury and during development of atherosclerosis[39]. The thrombin receptors PARs are also upregulated in VSMCs during injury. In this study, we showed that Orai3, the unique component of thrombin-activated Orai1/3 channels, is upregulated in synthetic VSMCs in vitro and in medial and neointimal VSMCs in vivo in a rat model of vascular injury. We also show that preventing Orai3 upregulation during carotid injury using lentiviral particles encoding shRNA reverses the increase in Orai3 protein levels 14 days post injury, inhibits LRC channel activation in medial and neointimal VSMC and inhibits neointima formation. We and others previously showed that STIM1 and Orai1 are important mediators in neointima formation[16, 26–29]. Orai3 or Orai3-containing channels could represent a potential target for treatment of VSMC remodeling during vascular occlusive diseases and might represent a better target than Orai1 or STIM1 since STIM1/Orai1-mediated CRAC is ubiquitous and prominently functional in many tissues, including immune cells and skeletal muscle; the major defect in Orai1-deficient patients and mice is severe immunodeficiency and skeletal muscle hypotonia[40].

We show that STIM1 is required downstream of LTC_4 action during the activation of this novel LRC channel. This fact, along with the high Ca^{2+} selectivity of this channel, suggests that STIM1 might be a component of the Orai1/3 channel complex. Indeed, recent data from the Prakriya group showed that STIM1endows -otherwise non-selective- Orai Ca^{2+} channels with high Ca^{2+} selectivity[41]. Several questions remain to be answered by future structural studies: how LTC_4 triggers Orai1/3 channel activation and whether this is a direct action? If it is through direct action, what are the domains in STIM/Orai that are involved in this interaction? What is the exact stoichiometry of LTC_4-activated channels in VSMC? The answer to these questions and others will likely help in the targeting of this channel for the purpose of therapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Nonstandard Abbreviations

CRAC	calcium release-activated calcium
STIM1	Stromal interacting molecule1
SOAR/CAD	STIM Orai activating region/CRAC activating domain
PLC	Phospholipase C
VSMC	vascular smooth muscle cell
SOCE	store-operated calcium entry
TRPC channels	transient receptor potential canonical channels
AA	Arachidonic Acid
LTC_4	leukotrieneC_4
PDGF	platelet-derived growth factor

References

1. Berridge MJ. Calcium microdomains: Organization and function. Cell calcium. 2006; 40:405–412. [PubMed: 17030366]
2. Clapham DE. Calcium signaling. Cell. 2007; 131:1047–1058. [PubMed: 18083096]
3. Putney JW Jr. A model for receptor-regulated calcium entry. Cell calcium. 1986; 7:1–12. [PubMed: 2420465]
4. Bird GS, Aziz O, Lievremont JP, Wedel BJ, Trebak M, Vazquez G, Putney JW Jr. Mechanisms of phospholipase c-regulated calcium entry. Current molecular medicine. 2004; 4:291–301. [PubMed: 15101686]
5. Hoth M, Penner R. Depletion of intracellular calcium stores activates a calcium current in mast cells. Nature. 1992; 355:353–356. [PubMed: 1309940]
6. Roos J, DiGregorio PJ, Yeromin AV, Ohlsen K, Lioudyno M, Zhang S, Safrina O, Kozak JA, Wagner SL, Cahalan MD, Velicelebi G, Stauderman KA. Stim1, an essential and conserved component of store-operated ca2+ channel function. The Journal of cell biology. 2005; 169:435–445. [PubMed: 15866891]
7. Liou J, Kim ML, Heo WD, Jones JT, Myers JW, Ferrell JE Jr, Meyer T. Stim is a ca2+ sensor essential for ca2+-store-depletion-triggered ca2+ influx. Current biology : CB. 2005; 15:1235–1241. [PubMed: 16005298]
8. Feske S, Gwack Y, Prakriya M, Srikanth S, Puppel SH, Tanasa B, Hogan PG, Lewis RS, Daly M, Rao A. A mutation in orai1 causes immune deficiency by abrogating crac channel function. Nature. 2006; 441:179–185. [PubMed: 16582901]
9. Vig M, Peinelt C, Beck A, Koomoa DL, Rabah D, Koblan-Huberson M, Kraft S, Turner H, Fleig A, Penner R, Kinet JP. Cracm1 is a plasma membrane protein essential for store-operated ca2+ entry. Science. 2006; 312:1220–1223. [PubMed: 16645049]
10. Prakriya M, Feske S, Gwack Y, Srikanth S, Rao A, Hogan PG. Orai1 is an essential pore subunit of the crac channel. Nature. 2006; 443:230–233. [PubMed: 16921383]
11. Soboloff J, Madesh M, Gill DL. Sensing cellular stress through stim proteins. Nat Chem Biol. 2011; 7:488–492. [PubMed: 21769090]
12. Park CY, Hoover PJ, Mullins FM, Bachhawat P, Covington ED, Raunser S, Walz T, Garcia KC, Dolmetsch RE, Lewis RS. Stim1 clusters and activates crac channels via direct binding of a cytosolic domain to orai1. Cell. 2009
13. Yuan JP, Zeng W, Dorwart MR, Choi YJ, Worley PF, Muallem S. Soar and the polybasic stim1 domains gate and regulate orai channels. Nature cell biology. 2009; 11:337–343.

14. Muik M, Fahrner M, Derler I, Schindl R, Bergsmann J, Frischauf I, Groschner K, Romanin C. A cytosolic homomerization and a modulatory domain within stim1 c-terminus determine coupling to orai1 channels. The Journal of biological chemistry. 2009
15. Kawasaki T, Lange I, Feske S. A minimal regulatory domain in the c terminus of stim1 binds to and activates orai1 crac channels. Biochemical and biophysical research communications. 2009
16. Potier M, Gonzalez JC, Motiani RK, Abdullaev IF, Bisaillon JM, Singer HA, Trebak M. Evidence for stim1- and orai1-dependent store-operated calcium influx through icrac in vascular smooth muscle cells: Role in proliferation and migration. FASEB journal : official publication of the Federation of American Societies for Experimental Biology. 2009; 23:2425–2437. [PubMed: 19364762]
17. Trebak M. Plc: Johnny-come-lately to orai and the ups and downs of calcium signalling. The Journal of physiology. 2011; 589:5337–5338. [PubMed: 22086246]
18. Motiani RK, Abdullaev IF, Trebak M. A novel native store-operated calcium channel encoded by orai3: Selective requirement of orai3 versus orai1 in estrogen receptor-positive versus estrogen receptor-negative breast cancer cells. The Journal of biological chemistry. 2010; 285:19173–19183. [PubMed: 20395295]
19. Motiani RK, Zhang X, Harmon KE, Keller RS, Matrougui K, Bennett JA, Trebak M. Orai3 is an estrogen receptor alpha-regulated ca2+ channel that promotes tumorigenesis. FASEB journal : official publication of the Federation of American Societies for Experimental Biology. 2012
20. Shuttleworth TJ. Arachidonic acid, arc channels, and orai proteins. Cell calcium. 2009
21. Mignen O, Thompson JL, Shuttleworth TJ. Both orai1 and orai3 are essential components of the arachidonate-regulated ca2+-selective (arc) channels. The Journal of physiology. 2008; 586:185–195. [PubMed: 17991693]
22. Mignen O, Thompson JL, Shuttleworth TJ. Stim1 regulates ca2+ entry via arachidonate-regulated ca2+-selective (arc) channels without store depletion or translocation to the plasma membrane. The Journal of physiology. 2007; 579:703–715. [PubMed: 17158173]
23. Trebak M, Vazquez G, Bird GS, Putney JW Jr. The trpc3/6/7 subfamily of cation channels. Cell calcium. 2003; 33:451–461. [PubMed: 12765690]
24. Yuan JP, Zeng W, Huang GN, Worley PF, Muallem S. Stim1 heteromultimerizes trpc channels to determine their function as store-operated channels. Nature cell biology. 2007; 9:636–645.
25. House SJ, Potier M, Bisaillon J, Singer HA, Trebak M. The non-excitable smooth muscle: Calcium signaling and phenotypic switching during vascular disease. Pflugers Archiv : European journal of physiology. 2008; 456:769–785. [PubMed: 18365243]
26. Berra-Romani R, Mazzocco-Spezzia A, Pulina MV, Golovina VA. Ca2+ handling is altered when arterial myocytes progress from a contractile to a proliferative phenotype in culture. American journal of physiology. Cell physiology. 2008; 295:C779–790. [PubMed: 18596214]
27. Guo RW, Wang H, Gao P, Li MQ, Zeng CY, Yu Y, Chen JF, Song MB, Shi YK, Huang L. An essential role for stim1 in neointima formation following arterial injury. Cardiovasc Res. 2008
28. Aubart FC, Sassi Y, Coulombe A, Mougenot N, Vrignaud C, Leprince P, Lechat P, Lompre AM, Hulot JS. Rna interference targeting stim1 suppresses vascular smooth muscle cell proliferation and neointima formation in the rat. Mol Ther. 2008
29. Zhang W, Halligan KE, Zhang X, Bisaillon JM, Gonzalez-Cobos JC, Motiani RK, Hu G, Vincent PA, Zhou J, Barroso M, Singer HA, Matrougui K, Trebak M. Orai1-mediated i (crac) is essential for neointima formation after vascular injury. Circulation research. 2011; 109:534–542. [PubMed: 21737791]
30. Bisaillon JM, Motiani RK, Gonzalez-Cobos JC, Potier M, Halligan KE, Alzawahra WF, Barroso M, Singer HA, Jourd'heuil D, Trebak M. Essential role for stim1/orai1-mediated calcium influx in pdgf-induced smooth muscle migration. American journal of physiology. Cell physiology. 2010; 298:C993–1005. [PubMed: 20107038]
31. Martorell L, Martinez-Gonzalez J, Rodriguez C, Gentile M, Calvayrac O, Badimon L. Thrombin and protease-activated receptors (pars) in atherothrombosis. Thrombosis and haemostasis. 2008; 99:305–315. [PubMed: 18278179]
32. Raines EW. Pdgf and cardiovascular disease. Cytokine Growth Factor Rev. 2004; 15:237–254. [PubMed: 15207815]

33. Hirano K. The roles of proteinase-activated receptors in the vascular physiology and pathophysiology. Arteriosclerosis, thrombosis, and vascular biology. 2007; 27:27–36.
34. Prakriya M, Lewis RS. Separation and characterization of currents through store-operated crac channels and mg2+-inhibited cation (mic) channels. The Journal of general physiology. 2002; 119:487–507. [PubMed: 11981025]
35. McIntyre TM, Zimmerman GA, Prescott SM. Leukotrienes c4 and d4 stimulate human endothelial cells to synthesize platelet-activating factor and bind neutrophils. Proceedings of the National Academy of Sciences of the United States of America. 1986; 83:2204–2208. [PubMed: 3457383]
36. Coughlin SR. Protease-activated receptors in hemostasis, thrombosis and vascular biology. Journal of thrombosis and haemostasis : JTH. 2005; 3:1800–1814. [PubMed: 16102047]
37. Coughlin SR. Thrombin signalling and protease-activated receptors. Nature. 2000; 407:258–264. [PubMed: 11001069]
38. Abdullaev IF, Bisaillon JM, Potier M, Gonzalez JC, Motiani RK, Trebak M. Stim1 and orai1 mediate crac currents and store-operated calcium entry important for endothelial cell proliferation. Circulation research. 2008; 103:1289–1299. [PubMed: 18845811]
39. Schror K, Bretschneider E, Fischer K, Fischer JW, Pape R, Rauch BH, Rosenkranz AC, Weber AA. Thrombin receptors in vascular smooth muscle cells - function and regulation by vasodilatory prostaglandins. Thrombosis and haemostasis. 2010; 103:884–890. [PubMed: 20143010]
40. Feske S. Calcium signalling in lymphocyte activation and disease. Nat Rev Immunol. 2007; 7:690–702. [PubMed: 17703229]
41. McNally BA, Somasundaram A, Yamashita M, Prakriya M. Gated regulation of crac channel ion selectivity by stim1. Nature. 2012; 482:241–245. [PubMed: 22278058]

Novelty and Significance

What Is Known?

- The ubiquitous store-operated Orai1 Ca^{2+} channels show low expression in healthy quiescent vascular smooth muscle cells (VSMC) but their expression is increased during VSMC remodeling into a proliferative migratory phenotype.
- Activation of VSMC with the platelet-derived growth factor (PDGF), a VSMC mitogen, activates Orai1-mediated Ca^{2+} entry through a mechanism involving endoplasmic reticulum (ER) Ca^{2+} store depletion and subsequent interaction of the Ca^{2+} sensor STIM1 with Orai1.
- Molecular knockdown of Orai1 inhibits neointima formation in response to balloon injury in rat carotid arteries.

What New Information Does This Article Contribute?

- VSMC activation by thrombin activates a novel Ca^{2+} entry pathway that requires both Orai1 and its homolog Orai3. This pathway requires STIM1, but does not depend on ER Ca^{2+} store depletion.
- This novel Ca^{2+} entry pathway in VSMC is mediated by the intracrine actions of leukotriene C_4 (LTC_4) produced downstream of thrombin receptor stimulation.
- Balloon injury of rat carotid arteries increased Orai3 expression and manifestation of Ca^{2+} currents activated by LTC_4 dialysis in medial and neointimal VSMC; healthy medial VSMC show no LTC_4-activated currents.
- Knockdown of Orai3 in balloon-injured carotid arteries using lentivirus-encoding shRNA prevents Orai3 upregulation, inhibits LTC_4-activated currents and decreases neointima formation.

Orai1, a store-operated Ca^{2+} channel activated by ligation of phospholipase C (PLC)-coupled receptors, is required for neointima formation upon vascular injury. However, Orai1 is functional in many cell types and tissues which could complicate its use as a specific target in VSMC-related pathologies. Orai1 has two homologs Orai2 and Orai3; Orai2 is expressed exclusively in vertebrates while Orai3 is expressed exclusively in mammals. The role of Orai2 and Orai3 in the vascular system remained unknown. We describe a new role of Orai3 in VSMC Ca^{2+} signaling and remodeling. We show that Orai3 contributes to a novel heteromeric Orai1/3 Ca^{2+} entry channel in thrombin-activated VSMC. We found that Orai1/3 channel activation is store-independent and mediated by cytosolic LTC_4 produced downstream thrombin receptor. Furthermore, Orai3 expression and LTC_4-activated channel activity increase in VSMC upon rat carotid artery injury while knockdown of Orai3 in injured carotids inhibits Orai3 upregulation, LTC_4-activated channels and neointima formation. These findings suggest that Orai3 represents a novel drug target for controlling VSMC remodeling during vascular injury or disease, and that Orai3 may be a better target than the ubiquitous Orai1 channel.

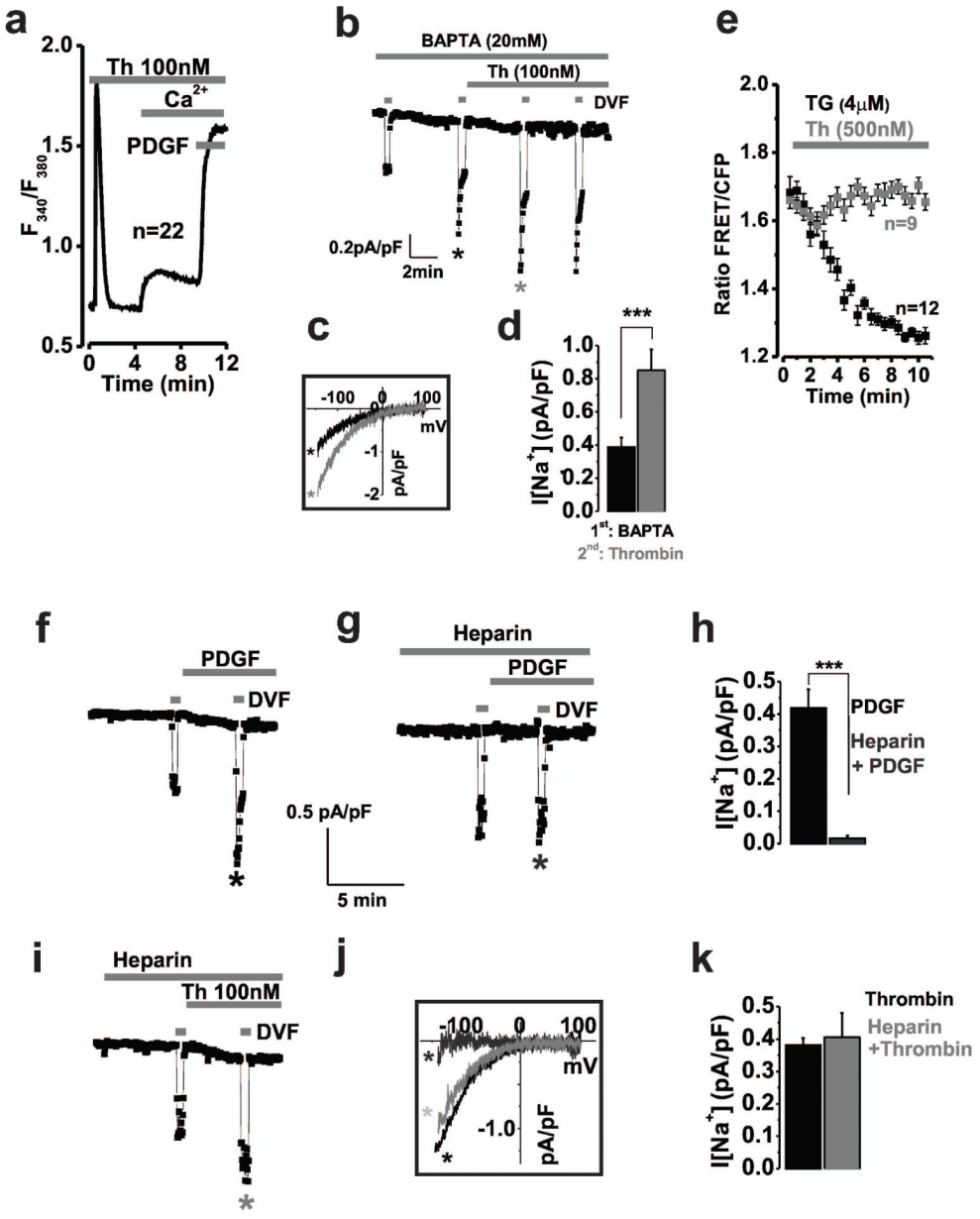

Figure 1. Thrombin-activated Ca^{2+} entry and currents are additive to SOCE and CRAC, and are store-independent

a; Ca^{2+} imaging experiments showing addivity between thrombin- (100nM) and PDGF- (100ng/mL) activated Ca^{2+} entry pathways. **b**; Whole- cell patch clamp electrophysiology shows additivity of CRAC currents (activated by dialysis of 20mM BAPTA through the patch pipette for 6min) and thrombin-activated currents (Na^+ I/V depicted in **c** and statistics in **d**). **e**; ER-Ca^{2+} levels were measured using the ER-targeted FRET sensor Cameleon-D1ER, before and after stimulation with maximal concentrations of thapsigargin (TG 4μM; **n=12**) and Thrombin (Th 500nM; **n=9**); only thapsigargin caused a significant decrease in ER Ca^{2+} levels. Whole-cell patch clamp electrophysiology showing the development of PDGF-activated CRAC currents with typical depotentiation in DVF solutions (PDGF 100ng/mL; **f**). Heparin (3mg/mL) dialysis through the patch pipette for 6 minutes completely abrogated PDGF-activated CRAC (**g, h**). Heparin dialysis failed to inhibit the

development of thrombin-activated currents (**i, k**). Na^+ I/V relationships taken from traces (**f, g, i**) where indicated by the color-coded asterisks are depicted in **j**.

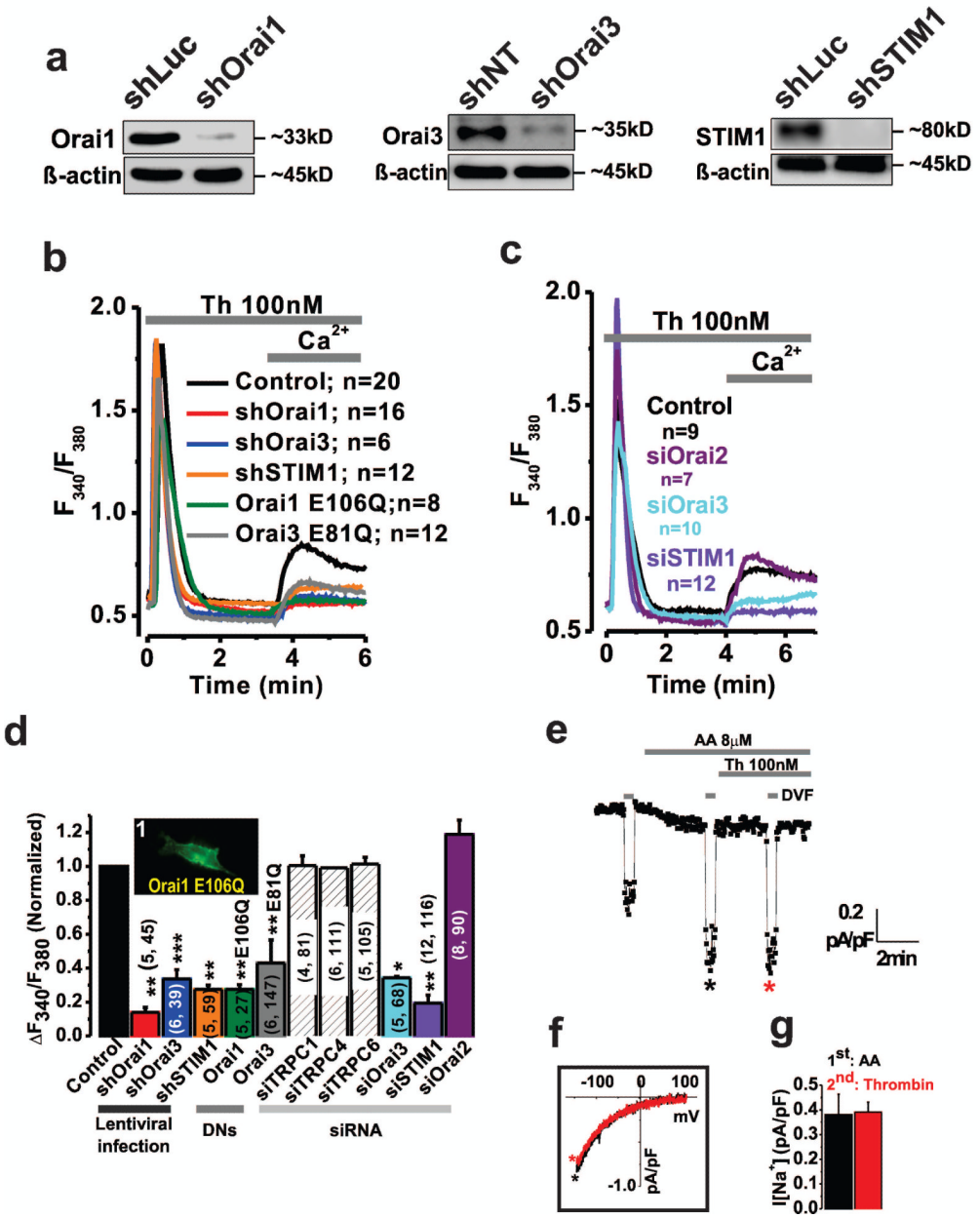

Figure 2. Thrombin-activated Ca^{2+} entry is mediated by STIM1, Orai1 and Orai3

Efficiency of STIM1, Orai1 and Orai3 protein knockdown after shRNA infection was documented by western blot (**a**). Representative Ca^{2+} imaging traces in response to 100nM thrombin in VSMCs infected for 7days with lentivirus-encoding a non-targeting shRNA (shNT, control for shOrai3) or an shRNA targeting fly luciferase (shLuc, control for shSTIM1 and shOrai1; see online methods for details), or shRNA targeting either Orai1, Orai3 or STIM1 (**b**); VSMCs were also transfected (and assayed after 36 hours) with either pore mutants of Orai1 (O1-E106Q), Orai3 (O3-E81Q) or GFP vectors (control) (**b**). All control traces (shNT, shLuc, GFP) show comparable Ca^{2+} entry to wild type cells and only one trace, shLuc is shown. A representative image showing membrane expression of Orai1-E106Q construct is shown in inset **d1**. Representative Ca^{2+} imaging traces in response to 100nM thrombin in VSMCs transfected with siRNA sequences targeting Orai2, Orai3 and

STIM1 and non-targeting control siRNA (**c**). **d**; Statistical summary on several independent Ca^{2+} imaging experiments, including experiments described in **b** and **c**. Statistics on Ca^{2+} imaging experiments evaluating the contributions of TRPC1/4/6 channels (the only isoforms expressed in rat VSMCs) to thrombin-activated Ca^{2+} entry are also shown (**d**). Whole-cell patch clamp electrophysiological recordings showing no additivity between arachidonic acid (AA; 8μM) and thrombin (100nM; **e–g**). For experiments depicted in **e**, I/V relationships are also shown (**f**). Origins of I/V curves on the current traces are indicated by the color-coded asterisks. The statistical summary is also included in **g**.

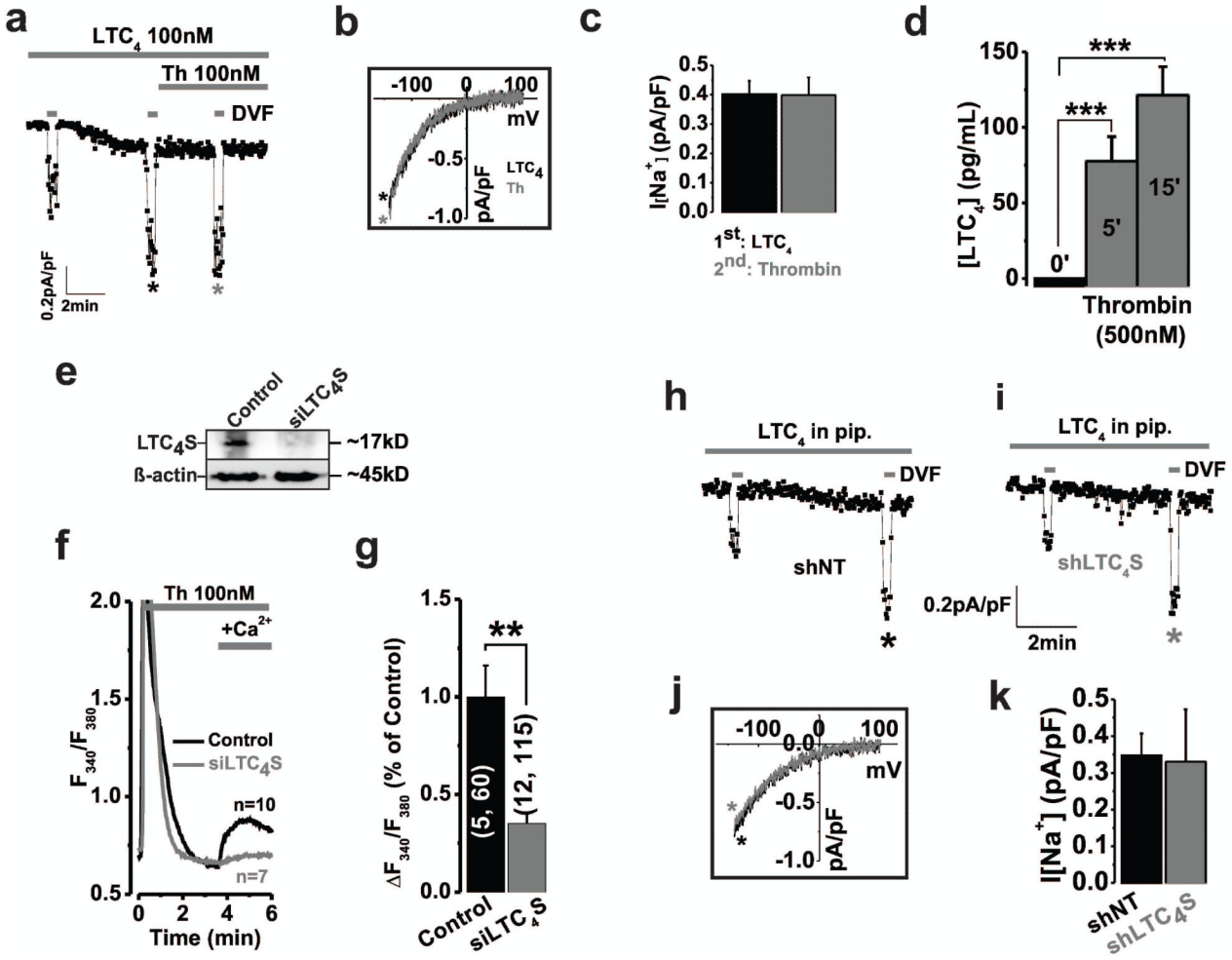

Figure 3. Thrombin-activated Ca^{2+} entry and currents are mediated through LTC_4 production

Whole-cell patch clamp electrophysiological recordings testing for additivity between LTC_4 and thrombin (**a–c**). I/V relationships and statistical summary are shown in **b** and **c** respectively. **d**; Competitive ELISA measurements of LTC_4 concentrations from VSMC cultures of either control cells (t=0) or cells stimulated with 500nM thrombin for a duration of 5 or 15 minutes. **e**; Cells transfected with siRNA against LTC_4S show significant knockdown of LTC_4S protein and abrogation of thrombin-activated Ca^{2+} entry (**f, g**). **f**; Representative Ca^{2+} imaging traces and statistical analysis (**g**) from cells transfected with either control siRNA or LTC_4S siRNA and stimulated with thrombin. Whole-cell patch clamp electrophysiology in VSMCs infected with lentivirus carrying either non targeting control (shNT) or LTC_4S shRNA ($shLTC_4S$) showed that dialysis of LTC_4 (100nM) through the pipette was able to activate currents indistinguishable from thrombin-activated currents in control cells and LTC_4S-depleted cells (**h, i**). Na^+ I/V relationships (**j**) and statistical analysis (**k**) are shown for LTC_4-activated currents. Origins of I/V curves on the current traces are indicated by the color-coded asterisks.

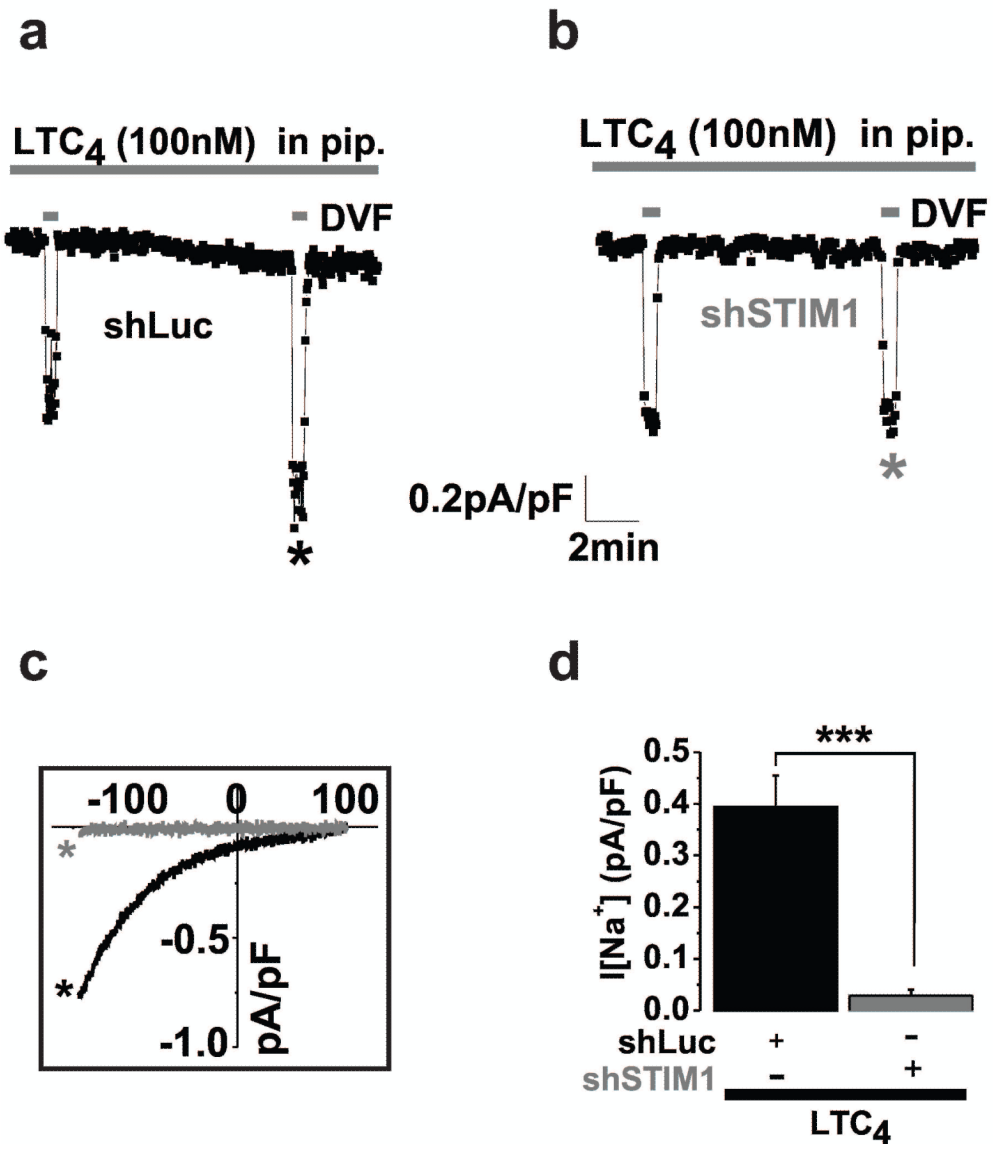

Figure 4. STIM1 is required for LTC_4-activated currents

Whole-cell patch clamp recordings of LTC_4- activated currents (**a–d**) in VSMCs infected with lentiviral vectors encoding either control shRNA against luciferase (shLuc) or STIM1 shRNA (shSTIM1). Depletion of STIM1 completely abrogated currents activated by inclusion of LTC_4 in the patch pipette (**b**). Na^+ I/V relationships are shown for LTC_4-activated currents (**c**). Statistics on current data are shown in **d**. Na^+ I/V relationships are taken from current traces where indicated by the color-coded asterisks.

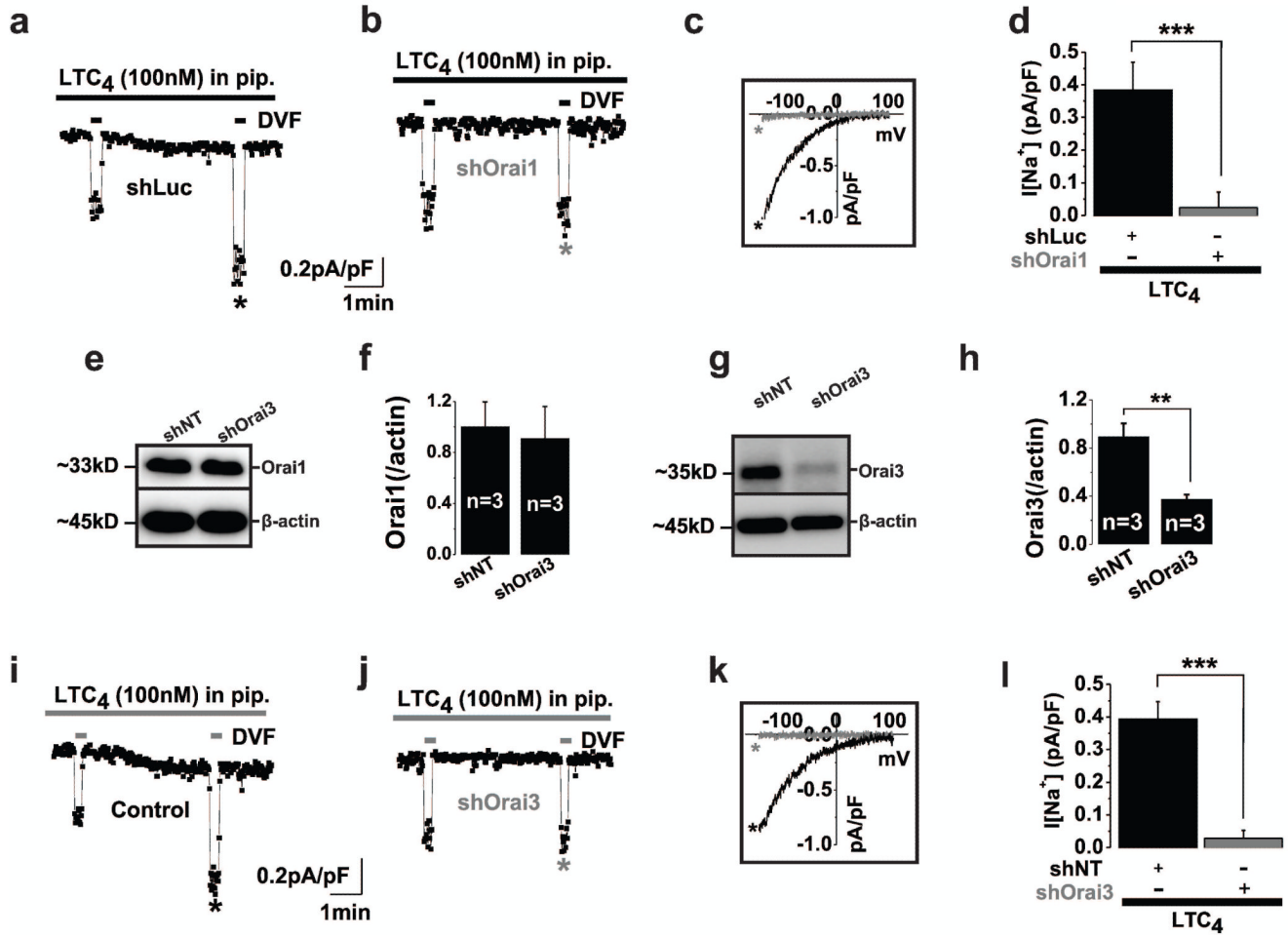

Figure 5. Orai1 and Orai3 are required for LTC_4-activated currents

Whole-cell patch clamp electrophysiology in VSMCs infected with lentivirus carrying either shRNA against luciferase (shLuc), or shRNA targeting Orai1 (shOrai1). Orai1 knockdown completely abrogated LTC_4-activated Na^+ currents (**b**) as compared to control (**a**). Na^+ I/V relationships (**c**) confirm the requirement of Orai1 for LTC_4-activated currents in VSMCs. Statistical analysis is shown in **d**. Representative western blots showing that shRNA targeting Orai3 does not affect Orai1 protein levels (**e, f**) while significantly abrogating Orai3 protein expression (**g, h**). Whole-cell patch clamp electrophysiology in VSMCs infected with lentivirus-encoding either non-targeting control shRNA (shNT) or shRNA targeting Orai3 (shOrai3). Orai3 knockdown completely abrogated LTC_4-activated currents (**j**) as compared to control (**i**). The I/V relationships are shown in **k**. Statistical analysis of patch clamp data is shown in **l**. Values for current densities represented as mean± range and number of independent recordings for shRNA Control, shRNA Orai1 and shRNA Orai3 are reported in Table 1.

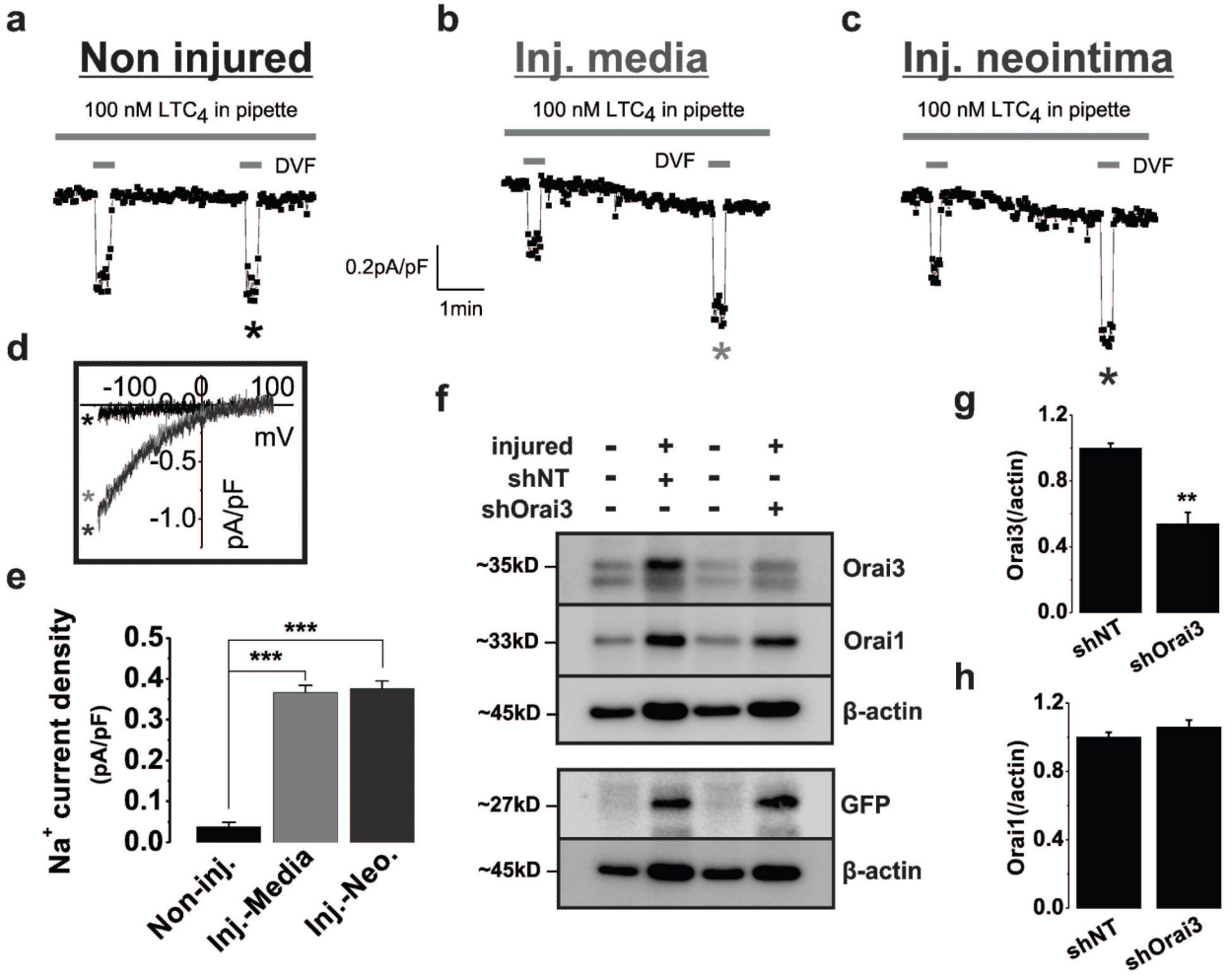

Figure 6. Orai3 and LRC currents are upregulated in VSMC after vascular injury

Whole-cell patch clamp electrophysiological recordings on VSMCs freshly isolated from media of non-injured carotids (**a**, n=4) or from either media or neointima of injured carotid arteries. Dialysis of LTC_4 through the patch pipette activated Ca^{2+} selective LRC currents only in VSMC isolated from either media (**b**) or neontima (**c**) of injured vessels 14 days post-injury. Na^+ I/V relationships are taken from data points where indicated by asterisks and shown in **d**. Statistical summary for this experiment is also shown in (**e**). Lentiviral infection with shRNA targeting Orai3 (shOrai3) after balloon injury prevented up-regulation of Orai3 in injured carotid artery with no significant effect on Orai1 **(f–h**; n=5**).** Control non-targeting shRNA (shNT) and Orai3 shRNA (shOrai3) lentiviruses efficiently infected carotid arteries as evidenced by GFP expression in the protein lysate of media and neointima from left (injured) carotid arteries 14 days after injury and infection (**f**); no GFP signal was detected in the protein lysate from the right (non-injured and non-infected) carotid artery.

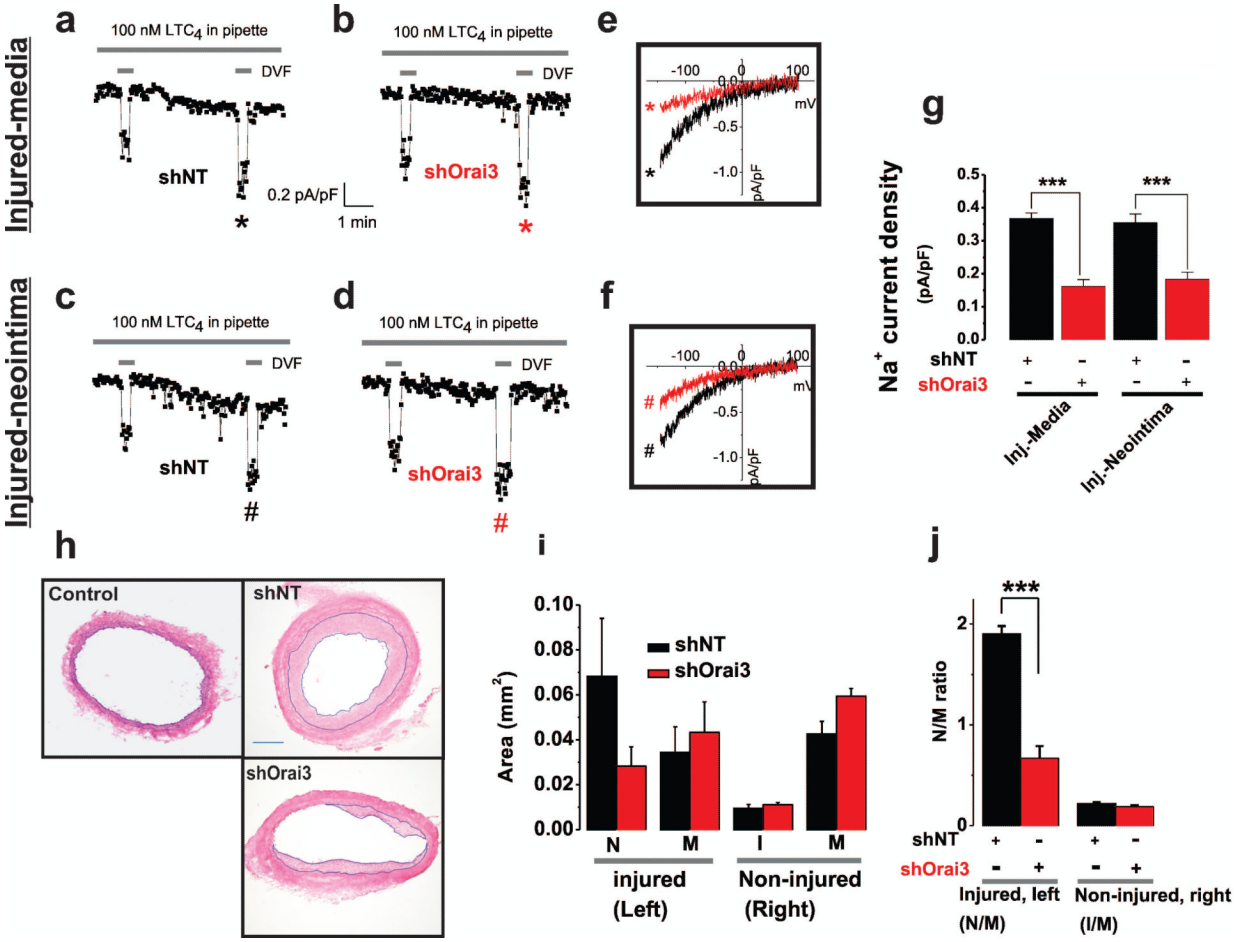

Figure 7. In vivo knockdown of Orai3 inhibits LRC currents and neointima formation

Whole-cell patch clamp electrophysiological recordings of VSMCs freshly isolated from the media (**a, b**) or neointima (**c, d**) of injured carotid arteries two weeks post injury and transduction treatment with viral particles carrying either a control shRNA sequence (shNT) or a sequence targeting Orai3 (shOrai3). As shown in figure 6 for injured vessels, carotid vessels injured and treated with shNT are characterized by the emergence of a Ca^{2+}-selective LRC current activated by intracytoplasmic LTC_4 in both medial (**a; n=4**) and neontimal VSMCs (**c; n=4**). This Ca^{2+} selective LRC current was reduced upon Orai3 knockdown with shRNA by ~55% in medial cells (**b; n=5**) and ~48% in neointimal cells (**d; n=5**). Na^+ I/V relationships are taken from traces where indicated by the color-coded signs and are shown in (**e, f**). Statistical summary is shown in (**g**). **h**; H&E staining on vessel cross-sections from control left carotid isolated from a sham-operated rat, and from injured left carotid arteries infected with either control shNT or shOrai3 (scale bar = 200μm). Fourteen days after injury, neointimal growth was evident in left carotids injured and infected with shNT compared to left control non-injured vessels from sham-operated animals. This neointima (N) was visibly inhibited by shOrai3 as compared to shNT. **i**; The neointimal (N), intimal (I) and medial (M) areas of the carotid cross sections were measured from left injured and virus-treated carotids and from right non-injured and non-infected carotids from the same animals using Image J software and statistical analyses on areas (**mm^2**) are shown. The media/neointima (N/M) ratios for left-injured and virus-treated carotids or intima/media (I/M) ratios of right non-injured and non-infected right carotids from the same animals (**j**) from 5 independent rats per condition are shown. Statistics on

Western blots of medial and neointimal VSMCs and quantification of neointima were performed on 5 rats per condition.

TABLE 1

Statistical analyses of all patch clamp experiments performed in the study organized by figure and figure panels, showing mean ± range of Ca^{2+} and Na^{+} currents, corresponding n number and p values. Groups comparisons are done on recordings performed the same day. Please note that when considering recordings performed on separate days, the n number is higher than reported (e.g. see table for recordings with LTC_4 on VSMC infected with control shRNA). AA=arachidonic acid; nd=not determined. P values obtained from data not included in this table are also listed in Online Table II.

Fig.		Experiment	Stimulus	I[Ca^{2+}] (pA/pF)	n	I[Na^{+}] (pA/pF)	n	p value
1	**b**	***Additivity***	BAPTA (1st)	0.107 ± 0.038	5	0.387 ± 0.057	5	1st vs 2nd; p=0
		1st→2nd	Th (2nd)	0.224 ± 0.080	5	0.851 ± 0.125	5	
		Heparin Dialysis						
	f	no heparin	PDGF	0.118 ± 0.032	4	0.418 ± 0.058	4	
	g	heparin	PDGF	0.013 ± 0.016	4	0.015 ± 0.009	4	f vs g; p=0
	i	heparin	Th	0.109 ± 0.032	4	0.406 ± 0.073	4	con. vs i; p=0.60017
2	**e**	***Additivity***	AA (1st)	0.102 ± 0.038	6	0.381 ± 0.082	5	
		1st→2nd	Th (2nd)	0.103 ± 0.042	5	0.391 ± 0.040	4	1st vs 2nd; p=0.64243
3	**a**	***Additivity***	LTC_4 (1st)	0.097± 0.031	5	0.401± 0.046	5	
		1st→2nd	Th (2nd)	0.108± 0.034	4	0.398± 0.061	4	1st vs 2nd; p=0.85552
		shLTC$_4$S Knockdown						
	h	shNT	LTC_4 in pip.	nd	nd	0.347 ± 0.060	3	
	i	shLTC4S	LTC_4 in pip.	nd	nd	0.330 ± 0.143	3	h vs i; p=0.72379
4		***STIM1 knockdown***						
	a	shLuc	LTC_4 in pip.	0.097 ± 0.026	3	0.403 ± 0.061	3	
	b	shSTIM1	LTC_4 in pip.	0.016 ± 0.013	5	0.017 ± 0.012	4	a vs b; p=0
5		***Orai1 knockdown***						
	a	shLuc	LTC_4 in pip.	0.104 ± 0.023	4	0.384 ± 0.085	4	
	b	shOrai1	LTC_4 in pip.	0.016 ± 0.014	5	0.024 ± 0.047	3	a vs b; p=0.00004
		Orai3 knockdown						
	i	shNT	LTC_4 in pip.	0.100 ± 0.027	3	0.394 ± 0.053	3	
	j	shOrai3	LTC_4 in pip.	0.021 ± 0.041	5	0.028 ± 0.024	5	i vs j; p=0.00002

Fig.		Experiment	Stimulus	I[Ca^{2+}] (pA/pF)	n	I[Na^{+}] (pA/pF)	n	p value
6		***Freshly Isolated Cells***						
	a	Non-injured	LTC_4 in pip.	0.020 ± 0.004	4	0.038 ± 0.011	4	
	b	Injured-media	LTC_4 in pip.	0.094 ± 0.007	5	0.366 ± 0.018	5	a vs b; p=0.00004
	c	Injured-neointima	LTC_4 in pip.	0.092 ± 0.009	5	0.376 ± 0.019	5	a vs c; p=0.00001
7		***Freshly Isolated Cells***						
		Injured-media						
	a	shNT	LTC_4 in pip.	0.090 ± 0.009	4	0.368 ± 0.017	4	
	b	shOrai3	LTC_4 in pip.	0.038 ± 0.006	5	0.162 ± 0.020	5	a vs b; p=0.00013
		Injured- neointima						
	c	shNT	LTC_4 in pip.	0.102 ± 0.011	4	0.355 ± 0.026	4	
	d	shOrai3	LTC_4 in pip.	0.047 ± 0.004	5	0.184 ± 0.021	5	c vs d; p=0.00016
S2	**e**	***Additivity***	Th (1st)	0.099 ± 0.035	6	0.385 ± 0.058	5	
		1st→2nd	AA (2nd)	0.101± 0.045	5	0.399 ± 0.062	5	1st vs 2nd; p=0.41261
S3	**a**	***Additivity***	LTB4 (1st)	0.015± 0.017	5	0.015± 0.016	5	
		1st→2nd	Th (2nd)	0.103± 0.023	5	0.402± 0.051	5	1st vs 2nd; p=0
	d	***LTC_4in bath sol.***	LTC_4 in bath	0.012± 0.002	5	0.015± 0.003	5	
		siLTC_4S Knockdown						
	h	siControl	Thrombin	0.108 ± 0.017	3	0.375 ± 0.077	3	
	i	siLTC_4S	Thrombin	0.047 ± 0.016	5	0.158 ± 0.081	5	h vs i; p=0.00003
		shLTC_4S Knockdown						
	l	shNT	Thrombin	nd	nd	0.378 ± 0.213	3	
	m	shLTC_4S	Thrombin	nd	nd	0.120 ± 0.103	3	l vs m; p=0.02491
S4		***STIM1 knockdown***						
	a	shLuc	BAPTA	0.106 ±0.041	3	0.396 ±0.053	3	
	b	shSTIM1	BAPTA	0.014 ± 0.015	6	0.018 ± 0.014	5	a vs b; p=0
S5		***Orai1 knockdown***						
	a	shLuc	BAPTA	0.110 ± 0.025	4	0.401 ± 0.058	4	
	b	shOrai1	BAPTA	0.013 ± 0.016	5	0.032 ± 0.040	5	a vs b; p=0

Fig.		Experiment	Stimulus	I[Ca^{2+}] (pA/pF)	n	I[Na^{+}] (pA/pF)	n	p value
		Orai3 knockdown						
	e	shNT	BAPTA	0.102 ± 0.028	3	0.410 ± 0.062	3	
	f	shOrai3	BAPTA	0.094 ± 0.023	3	0.408 ± 0.059	3	e vs f; p=0.70322
S7		***Freshly Isolated Cells***						
	a	Non-injured	BAPTA	0.018 ± 0.005	4	0.080 ± 0.015	4	
	b	Injured-media	BAPTA	0.098 ± 0.007	5	0.400 ± 0.029	5	a vs b; p=0
	c	Injured-neointima	BAPTA	0.094 ± 0.007	5	0.404 ± 0.021	5	a vs c; p=0