Regulation and function of Ca$_{v}$3.1 T-type calcium channels in IGF-I-stimulated pulmonary artery smooth muscle cells

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Pluteanu F, Cribbs LL. Regulation and function of Ca$_{v}$3.1 T-type calcium channels in IGF-I-stimulated pulmonary artery smooth muscle cells. Am J Physiol Cell Physiol 300: C517–C525, 2011. First published December 9, 2010; doi:10.1152/ajpcell.00107.2010.—Arterial smooth muscle cells enter the cell cycle and proliferate in conditions of disease and injury, leading to adverse vessel remodeling. In the pulmonary vasculature, diverse stimuli cause proliferation of pulmonary artery smooth muscle cells (PASMCs), pulmonary artery remodeling, and the clinical condition of pulmonary hypertension associated with significant health consequences. PASMC proliferation requires extracellular Ca$^{2+}$ influx that is intimately linked with intracellular Ca$^{2+}$ homeostasis. Among the primary sources of Ca$^{2+}$ influx in PASMCs is the low-voltage-activated family of T-type Ca$^{2+}$ channels; however, up to now, mechanisms for the action of T-type channels in vascular smooth muscle cell proliferation have not been addressed. The Ca$_{v}$3.1 T-type Ca$^{2+}$ channel mRNA is upregulated in cultured PASMCs stimulated to proliferate with insulin-like growth factor-I (IGF-I), and this upregulation depends on phosphatidylinositol 3-kinase/Akt signaling. Multiple stimuli that trigger an acute rise in intracellular Ca$^{2+}$ in PASMCs, including IGF-I, also require the expression of Ca$_{v}$3.1 Ca$^{2+}$ channels for their action. IGF-I also led to cell cycle initiation and proliferation of PASMCs, and, when expression of the Ca$_{v}$3.1 Ca$^{2+}$ channel was knocked down by RNA interference, so were the expression and activation of cyclin D, which are necessary steps for cell cycle progression. These results confirm the importance of T-type Ca$^{2+}$ channels in proper progression of the cell cycle in PASMCs stimulated to proliferate by IGF-I and suggest that Ca$^{2+}$ entry through Ca$_{v}$3.1 T-type channels in particular interacts with Ca$^{2+}$-dependent steps of the mitogenic signaling cascade as a central component of vascular remodeling in disease.

INTRACELLULAR CALCIUM PLAYS a key role in specialized functions of vascular smooth muscle cells (VSMCs), ranging from the regulation of gene expression to the maintenance of vascular tone. Voltage-gated Ca$^{2+}$ channels (Ca$_{v}$) are a primary source of calcium influx in VSMC; however, the precise roles of the different classes of Ca$_{v}$2+ channels have not been fully defined. The voltage-activated L- and T-type calcium channels are present in numerous VSMC preparations, and, whereas the high-voltage-activated L-type channels are an established target for antihypertensive agents, the functional role of T-type Ca$^{2+}$ channels is largely unknown. In the cardiovascular system, two T-type Ca$^{2+}$ channels are present referred to as Ca$_{v}$3.1 and Ca$_{v}$3.2 (26). Although T-type channels may have a minimal contribution in normal VSMCs, it is becoming increasingly clear that this novel class of Ca$^{2+}$ channels is regulated in pathological conditions in the heart and vascular system (5, 16, 18, 20, 23, 24, 33).

Previously, T-type Ca$^{2+}$ channels were identified in pulmonary arteries and pulmonary artery smooth muscle cell (PASMC) preparations (30), where the Ca$_{v}$3.1 channel was shown to influence PASMC proliferation in culture, and the same study implicated Ca$_{v}$3.1 channels in cell cycle progression. Early studies measuring low-voltage-activated (LVA) T-type Ca$^{2+}$ currents from developing mouse oocytes (10) or sea urchin embryos (9) also suggested that these channels are uniquely involved in cell cycle progression. Similar observations were made in myocytes from newborn ventricular muscle (14) and VSMCs cultured from rat aorta (21). Additional studies in cultured VSMCs have confirmed that cell cycle progression and proliferation require calcium influx through multiple mechanisms that are not yet fully understood (2).

The proliferation of PASMCs is a key factor in the pathogenesis of diseases that involve vessel remodeling, such as pulmonary hypertension. In pathological conditions, growth factors, cytokines, and chemokines signal PASMC apoptosis, migration, and proliferation (15). Insulin-like growth factor-I (IGF-I) is one such factor produced both in vascular endothelial cells and VSMCs, where, via interactions with its receptor or IGF binding protein, it activates multiple signal transduction pathways. IGF-I has been implicated in the development of atherosclerotic lesions via its effects on VSMC proliferation and migration (11), and beneficial effects of IGF-I inhibitors have been observed in animal models of vascular restenosis (37). In addition, IGF-I is a known cell cycle regulator in diverse cell types, and its effects are mediated by signal transduction, primarily via phosphatidylinositol 3-kinase (PI3K)/Akt or mitogen-activated protein kinase (MAPK) pathways.

Because of the putative role of T-type Ca$^{2+}$ channels in cell cycle and cell proliferation and the importance of understanding the underlying mechanisms of PASMC proliferation in diseases that involve vessel remodeling, we investigated the regulation of T-type Ca$^{2+}$ channels in cultured PASMCs stimulated to proliferate with IGF-I. IGF-I leads to increased levels of the Ca$_{v}$3.1 T-type channel mRNA that are largely influenced by the PI3K/Akt signal transduction pathway. Moreover, RNA interference studies showed that cell cycle stimulation, as evidenced by indexes of PASMC proliferation and the production and activation of cyclins, is dependent on the expression of Ca$_{v}$3.1 Ca$^{2+}$ channels. Acutely stimulated rises in intracellular Ca$^{2+}$ by membrane depolarization, angiotensin II (ANG II), or IGF-I were also dependent on the expression of Ca$_{v}$3.1 channels. These results suggest that, in PASMCs, T-type Ca$^{2+}$ channel expression could be upregulated in pathological conditions and play a regulatory role in pulmonary artery remodeling and pulmonary hypertension.
MATERIALS AND METHODS

Reagents. IGF-I was purchased either from R&D Systems (Minneapolis, MN) or Invitrogen (Carlsbad, CA); ANG II was from Tocris (Ellisville, MO); and inhibitors PD-98059 and LY-294002 were from Ascent Scientific (Princeton, NJ). Constitutively active (caMEK1) and dominant-negative MAPK/ERK kinase (MEK1) (3) and Akt adenoviruses were provided by Dr. Jody Martin, and fura-2 AM was from Invitrogen/Molecular Probes.

PASMC culture. PASMCs were prepared from pulmonary arteries of young adult Sprague-Dawley rats. Animals were anesthetized, and the heart and lung were removed and kept in low-calcium physiological saline solution on ice. Intralobular arterioles (3rd and 4th branches) were dissected, cleaned of adventitia, and enzymatically dissociated in two steps: one incubation in 1 mg/ml collagenase D (Roche) to remove the remaining adventitia and endothelial cells, followed by a second digestion with 2 mg/ml collagenase and 0.5 mg/ml elastase (Roche) for 60 min, 37°C. Arteries were mechanically triturated with a polished Pasteur pipette, and resuspended in smooth muscle cell basal medium (Lonza). After 3–5 days, the medium was changed to 0.1% serum to eliminate fibroblasts, and after 3 days serum was added back. When confluent, the cells were passaged and expanded for freezing. Upon thawing, PASMCs were used for experiments from passage 3 to 9.

RNA interference. A 29-nucleotide target for short hairpin RNA (shRNA) knockdown of the rat CaV3.1 T-type Ca2+ channel was identified (5'-TGCTGGTCTACGGTCTCTCTTCATCATT-3') and incorporated into complementary shRNA oligonucleotides (Integrated DNA Technologies), which were cloned into RNAi-Ready pSIREN-DNR-DsRed-Express shuttle vector, and the recombinant shuttle vector was then used to construct the “AdX-Cav3.1sh” adenovirus using the Knockout Adenoviral RNAi System 2 (BD Clontech). Adenoviral stocks were amplified in human embryonic kidney-293 cells, and lysates were purified on cesium chloride gradients. Purified adenovirus was used to infect PASMCs with a multiplicity of infection of 100, which resulted in >90% infection.

Real-time reverse transcriptase polymerase chain reaction. Quantitative real-time reverse transcriptase polymerase chain reaction (RT-PCR) was used to quantify mRNA expression in PASMCs. Following various treatments, DNase-free RNA was prepared using RNeasy Mini Kit, according to the manufacturer’s protocol (Qiagen), and 1 μg was used for cDNA synthesized using iScript cDNA Synthesis Kit (BioRad). cDNA was used as a template for PCR in a reaction containing SYBR Green Master Mix (Fermentas) and 10 μM of each primer, on a 7300 Sequence Detection System (ABI). Relative quantification was done according to the ΔΔCt (comparative threshold cycle) method, where input was normalized to 18s rRNA. Statistical analyses were carried out based on dCt values using Student’s t-test as specified. Primer pairs were synthesized (Integrated DNA Technologies) (rat CaV3.1: 5’-CTCTGACAGGAGTGCTCCT-3’; 5’-TGCGGGGTCTACGAAGGAC-3’; rat CaV3.2: 5’-GGAATCT-3’; 5’-ATACATCTTTCCAGG-3’). Primer assays for 18s RNA, IGF receptor, cyclin D1, cyclin E1, and cyclin A2 were purchased from SA Biosciences.

Immunostaining. PASMCs were fixed with 4% paraformaldehyde, permeabilized with 0.5% Triton X-100, and blocked with 1% normal goat serum before incubation with primary antibodies: anti-Cav3.1 and -Cav3.2 smooth muscle actin SM-22 (Sigma). Cells were permeabilized with 0.5% Triton X-100 and blocked with 1% normal goat serum before incubation with primary antibodies: anti-Cav3.1 and -Cav3.2 smooth muscle actin SM-22 (Sigma). Secondary antibody was labeled with Alexa Fluor 488 or Alexa Fluor 568 (Invitrogen), and coverslips were mounted using ProLong Gold antifade reagent with 4,6-diamidino-2-phenylindole (Invitrogen) for nuclear counterstain. To quantify staining for CaV3.1, fluorescence intensity measurements were done using Image J software.

Intracellular calcium measurements. PASMCs were plated on 15-mm glass coverslips coated with gelatin and loaded with 4 μM fura-2 AM (Invitrogen), for 30 min at 37°C in 0.1% BSA Krebs solution (in mM: 135 NaCl, 5.9 KCl, 2 CaCl2, 1 MgCl2, 10 HEPES, 10 t-glucose, pH 7.4), followed by 30 min of unloading in the same solution, which allows the hydrolysis of AM group. After unloading, cells are mounted in a microscope chamber and perfused with Krebs solution at room temperature at 1 ml/min. Cells are visualized with a Nikon inverted microscope, excitation wavelength is appropriately controlled by a monochromator (Photonics, LPS-150), and images are captured with a Hamamatsu camera at 1 frame/s, both controlled by SimplePCI software (Compix). The application system was positioned close to the recording field, so that the solution exchange occurred within 10 s. PASMCs were perfused with high K+ Krebs solution (in mM: 82 NaCl, 59 KCl, 2 CaCl2, 1 MgCl2, 10 HEPES, 10 t-glucose, pH 7.4), or Ca2+-free Krebs solution (in mM: 135 NaCl, 5.9 KCl, 3 MgCl2, 1 EGTA, 10 HEPES, 10 t-glucose, pH 7.4). The recording protocol consisted of 30-s perfusion with normal Krebs (to record the baseline calcium), 30-s high K+ Krebs (to estimate the response to depolarizing pulses), wash with Krebs for 2 min, and then either 30-s application of ANG II (1 μM in normal Krebs) or 8-min application of IGF-I (100 ng/ml in normal Krebs). For experiments in Ca2+-free Krebs, both ANG II and IGF-I were prepared in Ca2+-free Krebs, and their application was preceded by 1–3 min of Ca2+-free Krebs. ANG II or IGF-I were applied only once per dish. Only cells that have shown positive response to high K+ Krebs were included in the analysis. Results are expressed as 340- to 380-nm fluorescence ratio, corrected for the background.

RESULTS

Vascular remodeling is a clinically relevant pathophysiological response, extensive pulmonary artery remodeling due to PASMC proliferation is a hallmark of pulmonary hypertension, and IGF-I is a known mediator of VSMC proliferation via its action on cell cycle progression and mitogenesis (32). When cultured rat PASMCs were synchronized in serum-free medium and then stimulated to proliferate with IGF-I, an approximate threefold increase of the T-type Ca2+ channel CaV3.1 mRNA was observed after 48 h (Fig. 1A), with no effect on CaV3.2 mRNA. Immunostaining for CaV3.1 showed predominating cytoplasmic staining (Fig. 1B; although membrane staining was also visible in confocal images, Fig. 1C), and cytoplasmic fluorescence intensity was also elevated approximately threefold after 48 h of IGF-I treatment (Fig. 1D).

Mitogenic signaling by IGF-I can involve the activation of numerous signal transduction pathways in the cell. Therefore, we investigated whether the stimulation of T-type channel CaV3.1 transcription is downstream from PI3K/Akt or MAPK pathways that are known to be activated by IGF-I. Quiescent, synchronized PASMC cultures were treated with IGF-I and in the presence of the inhibitor LY-294002, the upregulation of CaV3.1 mRNA was completely abolished at 48 h, and, similarly, inactivation with a dominant-negative Akt adenovirus fully blocked CaV3.1 upregulation. On the other hand, constitutively active myristoylated Akt adenovirus led to a 10-fold increase in basal CaV3.1 mRNA levels that was stimulated even further by IGF-I (Fig. 2A). Effects of ERK1/2–MAPK pathway signaling on the expression of CaV3.1 were tested in a similar fashion by
interfering with the activation of MEK1 (MAPK kinase, Fig. 2B). Treatment with IGF-I led to the typical two- to threefold increase in Ca\textsubscript{3.1} mRNA in PASMCs after 48 h, and this increase was partially but significantly blocked by the ERK1/2 inhibitor PD-98059. In the presence of either caMEK1 or dominant-negative MEK1 adenovirus, IGF-I stimulation resulted in modest upregulation of Ca\textsubscript{3.1} mRNA levels. Taken together, these experiments suggest that, in PASMCs, IGF-I

Fig. 1. Insulin-like growth factor-I (IGF-I) up-regulates T-type channels in cultured rat pulmonary artery smooth muscle cells (PASMCs). A: rat PASMCs were serum starved for 2–3 days before IGF-I stimulation. After 24 and 48 h in IGF-I, PASMCs were harvested for RNA purification and quantitative RT-PCR. RQ represents the relative quantification vs. time-matched control, unstimulated cells and is calculated based on the ΔΔCt (comparative threshold cycle) method, using 18S rRNA as endogenous control. Values are means ± SE. Statistical significance is calculated using paired t-test on ΔCt values. ***P < 0.001. B: immunostain of α-smooth muscle (α-SM) actin (SM-22, red), voltage-gated calcium channel (Ca\textsubscript{3.1}) (green), and nuclei counterstained with 4,6-diamidino-2-phenylindole (DAPI; blue) in unstimulated (control) and 48-h IGF-I stimulated PASMCs. Scale bar = 50 μM. C: confocal image of Ca\textsubscript{3.1} immunostain in 48-h IGF-I-treated PASMCs (as in B); scale bar represents 20 μm. D: averaged fluorescence intensity of Ca\textsubscript{3.1}-immunostained PASMCs with 48-h IGF-I treatment, normalized to control untreated PASMCs (as in B). Values are means ± SE. ***P < 0.001 for IGF-I (n = 61) vs. control (n = 41).

Fig. 2. Effect of signaling pathways activated by IGF-I on Ca\textsubscript{3.1} upregulation. A: constitutively active (ca, n = 4) and dominant-negative (dn, n = 4) protein kinase B (Akt) isoforms were overexpressed in cultured rat PASMCs using adenovirus constructs. Forty-eight hours after infection, PASMCs were stimulated with 100 ng/ml IGF-I for another 48 h and then harvested for RNA purification (n = 3). RQ of Ca\textsubscript{3.1} mRNA was calculated vs. control (β-galactosidase adenovirus infected, unstimulated) cells. The role of Akt activation in IGF-I-induced Ca\textsubscript{3.1} mRNA upregulation was also tested by blocking the pathway with 10 μM LY-294002 (n = 4). B: ca (n = 4) and dn (n = 4) MEK1 isoforms were overexpressed in cultured rat PASMCs, which were then exposed to 100 ng/ml IGF-I for an additional 48 h. The role of ERK1/2 activation in IGF-I-induced Ca\textsubscript{3.1} mRNA upregulation was also tested by blocking ERK1/2 with 10 μM PD-98059 (n = 4). Values are means ± SE. Statistical significance is calculated using paired t-test on ΔCt values. **P < 0.01, ***P < 0.001 vs. control (infected, unstimulated) PASMCs. #P < 0.05, ##P < 0.01, ###P < 0.001 reflect the effect of the inhibitory action on IGF-I stimulation.

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affects the upregulation of the Ca_{3.1} T-type Ca^{2+} channel largely via Akt signaling.

The relative contribution of T-type Ca^{2+} channels to calcium homeostasis in VSMCs remains questionable in normal conditions, but it may be relevant for quiescent and pathological events that require Ca^{2+} influx, such as cell cycle progression. For these experiments, efficient and specific knockdown of Ca_{3.1} was accomplished in PASMCs, which are relatively resistant to conventional transfection methods, using adenovirus-based RNA interference (Supplemental Figs. S1 and S2; the online version of this article contains supplemental data). When PASMCs were preinfected with the AdX-Cav3.1sh adenovirus and allowed a sufficient time for Ca_{3.1} knockdown, resting intracellular Ca^{2+} levels were significantly decreased compared with control (scrambled insert) “AdX-scr” adenovirus (Fig. 3). Furthermore, either membrane depolarization in high K^+ Krebs solution or treatment with ANG II, a potent vasoconstrictor that also signals VSMC proliferation and growth (8), resulted in a rise in intracellular Ca^{2+} that was significantly decreased in AdX-Cav3.1sh-infected PASMCs (Fig. 3A). The Ca^{2+} responses to ANG II were also reduced when mibebradil was used as a selective blocker of T-type Ca^{2+} channels, further suggesting at least a partial contribution of T-type channels to Ca^{2+} influx (Supplemental Fig. S4). The acute effects of IGF-I on cytosolic Ca^{2+} in PASMCs were also tested in control and Ca_{3.1} knockdown conditions, and Fig. 3B shows that, in control AdX-scr-infected PASMCs, IGF-I led to elevated Ca^{2+} levels within 2 min following exposure. Out of the total of PASMCs infected with either AdX-scr (101 cells) or AdX-Cav3.1-sh (152 cells), 68.3% of AdX-scr-infected cells showed a Ca^{2+} response to IGF-I, while only 11.2% of AdX-Cav3.1sh-infected cells responded. A gradual increase in intracellular Ca^{2+} during the 8-min IGF-I exposure was present in 74% of AdX-scr vs. 37% of AdX-Cav3.1sh-infected PASMCs. Importantly, when Ca^{2+} was omitted from the extracellular solution, no cytosolic Ca^{2+} increase was measurable in response to 100 ng/ml IGF-I (shaded line in Fig. 3B). Baseline resting Ca^{2+} before IGF-I application was reduced when Ca_{3.1} was knocked down, as was the peak Ca^{2+} response to acute IGF-I stimulation. Experiments in 0 mM Ca^{2+} confirmed that these acute effects of IGF-I are dependent on extracellular Ca^{2+} influx. Results of the above experiments are summarized in Fig. 3C.

Considering the known mitogenic effect of IGF-I and its observed effects on Ca_{3.1} expression, we tested the hypothesis that, in pathology, the upregulation of Cav3.1 is part of a positive feedback mechanism that potentiates proliferation induced by growth factors such as IGF-I. Proliferation of PASMCs was examined following IGF-I stimulation, by both BrdU incorporation and cell counting. Nuclear incorporation of BrdU increased significantly following 48 h of IGF-I treatment, and, when the Ca_{3.1} T-type Ca^{2+} channel was knocked down with AdX-Cav3.1sh, IGF-I-stimulated proliferation was significantly reduced (Fig. 4A). A similar effect of Ca_{3.1} shRNA-mediated knockdown was seen when PASMC proliferation was assessed by cell counting (Fig. 4B), and the inclusion of 3 μM mibebradil during IGF-I stimulation also abolished the increase in cell number ratio (Fig. 4B), suggesting that IGF-I-stimulated proliferation of PASMCs is dependent on the Ca_{3.1} T-type Ca^{2+} channel.

The mitogenic action of IGF-I is also closely associated with cell proliferation via the activation of cyclins. Production and activation of cyclins associated with the G1 phase (cyclins D, E, and A) provide checkpoints of early cell proliferation signaling (31). Considering that cyclin D is an early regulator of cell cycle initiation, and Ca^{2+} regulation is critical for cell
down of the Ca,3.1 channel prevented the IGF-I stimulation of cyclin D mRNA. Notably, in the same cultures, Ca,3.1 knockdown had no significant effect on expression of IGF receptor mRNA (also shown in Fig. 5).

When endogenous cyclin D1 was examined by immunostaining, nuclear translocation was observed within 5 h of treatment with IGF-I, and this effect was reduced when Ca,3.1 T-type channels were knocked down with AdX-Cav3.1sh (Fig. 6). For additional studies on cyclin D translocation in PASMCs, a fluorescent GFP-tagged cyclin D1 was incorporated into an adenoviral vector (“Ad-CycD-GFP”). PASMCs were infected, and the ratio of nuclear to cytoplasmic GFP was examined as an indicator of cyclin D activation (see Supplemental Fig. S5).

The transcription of cyclins D, E, and A is regulated in sequence as the cell cycle progresses from the G1 through the S phase, and, consistent with this, in PASMCs treated with IGF-I, we observed significant increases in cyclin E1 mRNA after 8 h, and for cyclin A2 mRNA at 24 h (Fig. 7). Similar to the impaired activation of cyclin D1 at early time points, knockdown of the Ca,3.1 T-type Ca2+ channel with AdX-Cav3.1sh also interfered with the production of these downstream cyclins.

DISCUSSION

In pathological conditions that invoke structural and functional vessel remodeling, cellular components of the arterial wall undergo numerous phenotypic changes. An important aspect of vascular remodeling processes, such as atherosclerosis and restenosis, is the transformation of VSMCs from a differentiated to a proliferative state. Increases in cytosolic Ca2+ by the concerted action of extracellular and intracellular Ca2+ sources can then contribute to changes in gene expression, cell cycle regulation, VSMC migration, and proliferation. Our results suggest that LVA T-type Ca2+ channels may play
a unique role in cell cycle/proliferation signaling in the vascular response to injury. Using RNA interference, we demonstrate that the Ca<sub>3.1</sub> Ca<sup>2+</sup> channel contributes to resting intracellular Ca<sup>2+</sup> levels in PASMCs, as well as to intracellular Ca<sup>2+</sup> increases stimulated with high K<sup>+</sup>, ANG II, or IGF-I. In addition to the acute effects of IGF-I on cytosolic Ca<sup>2+</sup> that require Ca<sub>3.1</sub> channel expression, more prolonged exposure of PASMCs to IGF-I stimulates cell proliferation and also leads to an increase in Ca<sub>3.1</sub> mRNA levels that is largely influenced by PI3K/Akt signaling. When Ca<sub>3.1</sub> expression was blocked, so was early cell cycle signaling by cyclin D, and the mitogenic action of IGF-I. Taken together, these results suggest a mechanism by which IGF-I can influence the proliferation of PASMCs via Akt-mediated increases in Ca<sub>3.1</sub> T-type channels, and their interaction with cell cycle signaling cascades.

**Contribution of the Ca<sub>3.1</sub> T-type channel to intracellular calcium.** Our laboratory’s earlier studies in the A7r5 aortic VSMC line using relatively selective concentrations of mibebradil implicated a role for T-type Ca<sup>2+</sup> channels in the generation of arginine vasopressin-stimulated Ca<sup>2+</sup> spiking, which may relate to the maintenance of peripheral resistance and blood pressure (4). However, up to now, the functional contributions of T-type Ca<sup>2+</sup> channels in VSMCs are poorly understood (6, 7), and they have not been investigated in conditions of vascular injury, where they could potentially be a therapeutic target. Here we present IGF-I as one mitogenic stimulus that may potentiate the effects of Ca<sub>3.1</sub> T-type Ca<sup>2+</sup> channels in cultured PASMCs.

We determined that infection with the AdX-Cav3.1sh adenovirus leads to specific knockdown of Ca<sub>3.1</sub> T-type channels, with no effect on transient receptor potential channel 1 (TrpC1) and TrpC6 mRNA expression or on L-type Ca<sup>2+</sup> currents (supplemental data). Therefore, we conclude that the reduction of induced Ca<sup>2+</sup> responses in AdX-Cav3.1sh-infected PASMCs is linked to the knockdown of T-type channels, as opposed to other known sources of Ca<sup>2+</sup> influx. We hypothesize that, in the disease setting in which growth factors...
and cytokines are activated, T-type Ca\(^{2+}\) channels become a significant source of Ca\(^{2+}\) as effectors of downstream signaling processes important for VSMC proliferation (see below). The upregulation of Ca\(_{3.1}\) mRNA expression by IGF-I may represent a potentiating effect of IGF-I on T-type Ca\(^{2+}\) channel influx that further enhances its putative signaling capabilities.

The RNA interference studies show that IGF-I-stimulated Ca\(^{2+}\) influx through Ca\(_{3.1}\) channels in PASMCs has a crucial, as yet undefined role in regulating intracellular Ca\(^{2+}\) and, hence, cellular function. Because of their transient nature and relatively small conductance, T-type Ca\(^{2+}\) channels are not likely to contribute directly to the observed cytosolic Ca\(^{2+}\) increases in VSMCs, but, rather, they may influence calcium homeostasis indirectly by interacting with other extracellular Ca\(^{2+}\) sources, such as voltage-gated L-type Ca\(^{2+}\) channels or canonical TRPCs (e.g., TRPC1 or TRPC6) (13, 36). T-type Ca\(^{2+}\) channel activity and growth factor-mediated signaling. IGF-I can have mitogenic effects on T-type channels are on the Cav3.2 isotype (25), which was relatively unaffected in PASMCs by IGF-I treatment and does not seem to be a significant source of Ca\(^{2+}\) during cell proliferation, since knocking down Ca\(_{3.1}\) alone in our experiments was sufficient to block the activation of cyclins. This emphasizes that the Ca\(_{3.1}\) and Ca\(_{3.2}\) T-type channel isotypes have distinct functional roles in the cell, as our laboratory has observed previously in cultured neonatal rat ventricular myocytes (29).

**Growth factor stimulation of PASMC proliferation.** In diseased, injured, and hypoxic blood vessels, the release of numerous growth factors and cytokines provides a signal for medial smooth muscle cell proliferation and vascular remodeling. We initially tested the effects of known mitogens (PDGF, IGF-I, and basic fibroblast growth factor) on Ca\(_{3.1}\) expression in both human and rat PASMCs, and chose IGF-I for further study based on its reproducible effects (Fig. 1). Although >90% of cultured PASMCs were smooth muscle cells based on positive immunostain for α-smooth muscle actin, we observed nonuniform responses to IGF-I. According to our results with FACS and BrdU incorporation only, a subset of the arrested cells responded by entering the cell cycle. This may be a reflection of previous reports, where the proliferation response of PASMCs differed, depending on their site of origin within the vessel (proximal or distal to the main branch pulmonary artery, and inner, middle vs. outer media) (12). In the latter study, heterogeneity in the response was at least partially attributed to alternative usage of cell signaling pathways (namely, PKC) by the different subpopulations of PASMCs, which underscores the need to further investigate the interactions between T-type Ca\(^{2+}\) channel activity and growth factor-mediated signaling.

**Role of T-type Ca\(^{2+}\) channels in IGF-I signaling pathways.** Our results support the idea that calcium entry through T-type channels in particular is targeted to specific cellular signaling mechanisms, which, in this case, are related to cell cycle and proliferation signaling. IGF-I can have mitogenic effects through two main signal transduction pathways: PI3K/Akt or MAPK/ERK1/2, which are activated in VSMCs and are relevant for proliferation and vascular remodeling. The same pathways may be activated by other growth factors and cytokines, and cross talk between these parallel signaling pathways can also occur to further affect cellular functions. Our experiments made use of adenoviruses encoding caMEK1 or dominant-negative variants of pathway components Akt or MEK1 (upstream from ERK1/2). The activation of Akt led to remarkable upregulation of Ca\(_{3.1}\) mRNA that was further increased by IGF-I stimulation, and blocking the PI3K pathway either with LY-294002 or dominant-negative Akt completely abolished the IGF-I effects. Therefore, PI3K/Akt signaling is an important intermediary of the upregulation of Ca\(_{3.1}\) mRNA in PASMCs by IGF-I and possibly other effectors that activate Akt.

In similar experiments to investigate MAPK signaling, caMEK1 showed no stimulation of Ca\(_{3.1}\) mRNA in the absence of IGF-I, and blocking ERK1/2 with PD-98059 only partially inhibited IGF-I-mediated stimulation of Ca\(_{3.1}\). Also, interfering with MEK1 activity had minimal effects on the regulation of Ca\(_{3.1}\) mRNA levels by IGF-I, suggesting that MAPK signaling plays a minimal role relative to Akt in the IGF-I stimulation of Ca\(_{3.1}\). Figure 2B. While IGF-I can activate both Akt and MEK1 signaling pathways, it is possible that, in these experiments, caMEK1 competes for a shared upstream activator, thereby diminishing the effects of Akt. Clearly, these two growth factor signaling mechanisms are utilized to different extents by VSMCs and can depend on cell type, stimulus, developmental stage, and pathological state (27). In aortic smooth muscle cells, both pathways are activated by IGF-I, and, whereas PI3K/Akt signaling primarily mediates cell migration (also an important aspect of vascular remodeling), MAPK signaling is more related to the proliferation response (17). Although we focused on the impact of IGF-I on proliferation, other consequences of Ca\(^{2+}\) influx via Ca\(_{3.1}\) channels in IGF-I-stimulated PASMCs warrant further investigation.

**Role of Ca\(_{3.1}\) channels in cell cycle signaling.** Since proliferation of VSMCs is a key underlying factor in multiple diseases involving vascular remodeling, inhibition of cell cycle signaling in VSMCs can be considered as a therapeutic target (2). Studies on experimental models of vascular injury have revealed complex, temporally regulated responses involving growth factor release, activation of protooncogenes, and cell cycle signal transduction cascades as potential sites of therapeutic intervention. Calcium is required during two main phases of the mammalian cell cycle: G\(_1\) during early G\(_1\), and G\(_2\) to S phase transition (19, 34). Consistent with this, proliferating PASMCs display more depolarized membrane potentials and elevated cytosolic Ca\(^{2+}\) (28). Still, the pathways of Ca\(^{2+}\) influx are not fully defined, and there is incomplete information about the Ca\(^{2+}\)-dependent mechanisms that control cell cycle and VSMC proliferation during vessel remodeling. Multiple lines of evidence have suggested that the LVA T-type Ca\(^{2+}\) channels are involved in cell cycle control, and our results suggest that, in PASMCs, T-type Ca\(^{2+}\) channels interact with cyclin D signaling to affect cell cycle activation during the early G\(_1\) phase. We observed elevated levels of cyclin D1 transcription in the initial hours following IGF-I
stimulation of serum-deprived PASMCs, along with cyclin D nuclear translocation that was dependent on expression of the Ca$_{3.1}$ Ca$^{2+}$ channel (Figs. 5 and 6). These experiments clearly implicate LVA Ca$^{2+}$ entry through Ca$_{3.1}$ T-type channels as an essential component of cyclin D signaling and cell cycle progression, in keeping with the observed inhibition of proliferation in AdX-Ca$^{3+}$-infected PASMCs. The knockdown of Ca$_{3.1}$ channels also had a negative influence on the expression of downstream cyclins A and E, which can involve Ca$^{2+}$/calmodulin and calcineurin as Ca$^{2+}$-dependent signaling intermediaries (35). While these observations support the importance of T-type Ca$^{2+}$ channels in proper cell cycle progression, it remains unclear whether reduction of these downstream cyclins is an effect of the observed inhibition of cyclin D, or, alternatively, additional Ca$^{2+}$-dependent processes rely on T-type Ca$^{2+}$ channel activity.

T-type Ca$^{2+}$ channels have been previously implicated in cell proliferation (22), and this is the first study to suggest a possible mechanism for T-type interaction in regulation of the cell cycle. The ability of Ca$^{2+}$ entry through T-type channels to carry out such specified signaling roles (as opposed to other known sources of Ca$^{2+}$ entry) suggests temporal or spatial compartmentalization of these channels in PASMCs, as has been suggested recently for T-type Ca$^{2+}$ channels in cardiomyocytes (23), and should be the subject of future investigations aimed toward understanding their relevance in VSMC pathology.

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DISCLOSURES

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