Low-Voltage–Activated (T-Type) Calcium Channels Control Proliferation of Human Pulmonary Artery Myocytes

David M. Rodman, Katherine Reese, Julie Harral, Brian Fouty, Songwei Wu, James West, Marloes Hoedt-Miller, Yuji Tada, Kai-Xun Li, Carlyne Cool, Karen Fagan, Leanne Cribbs

Abstract—While Ca\(^{2+}\) influx is essential for activation of the cell cycle machinery, the processes that regulate Ca\(^{2+}\) influx in this context have not been fully elucidated. Electrophysiological and molecular studies have identified multiple Ca\(^{2+}\) channel genes expressed in mammalian cells. Ca,3.x gene family members, encoding low voltage–activated (LVA) or T-type channels, were first identified in the central nervous system and subsequently in non-neuronal tissue. Reports of a potential role for T-type Ca\(^{2+}\) channels in controlling cell proliferation conflict. The present study tested the hypothesis that T-type Ca\(^{2+}\) channels, encoded by Ca,3.x genes, control pulmonary artery smooth muscle cell proliferation and cell cycle progression. Using quantitative RT/PCR, immunocytochemistry, and immunohistochemistry we found that Ca,3.1 was the predominant Ca,3.x channel expressed in early passage human pulmonary artery smooth muscle cells in vitro and in the media of human pulmonary arteries, in vivo. Selective blockade of Ca,3.1 expression with small interfering RNA (siRNA) and pharmacological blockade of T-type channels completely inhibited proliferation in response to 5% serum and prevented cell cycle entry. These studies establish that T-type voltage-operated Ca\(^{2+}\) channels are required for cell cycle progression and proliferation of human PA SMC. (Circ Res. 2005;96:864-872.)

Key Words: T-type calcium channel ■ pulmonary artery ■ smooth muscle cells ■ proliferation

At least 10 genes have been described that encode mammalian voltage-operated calcium channels (VOCCs).\(^1\) The channels fall into 3 broad families distinguished by the cell membrane potential at which they are active: (1) Ca,1, encoding high voltage–activated (HVA) L-type channels; (2) Ca,2, encoding HVA (or “mid” voltage-activated) P/Q-type, N-type, and R-type channels; and (3) Ca,3, encoding low voltage–activated (LVA) T-type channels. Electrophysiological studies have identified both HVA and LVA channels in vascular smooth muscle.\(^2,3\) The molecular identity (Ca,1.2, \(\alpha_{1C}\)) and role of HVA currents in initiation of contraction in vascular smooth muscle are well established. In contrast, the molecular identity and role of LVA currents in vascular smooth muscle are only now being defined.\(^4\)

Three genes encoding T-type Ca\(^{2+}\) channels, Ca,3.1 (\(\alpha_{1D}\)), Ca,3.2 (\(\alpha_{1H}\)), and Ca,3.3 (\(\alpha_{1I}\)), were cloned from brain.\(^5\) The channel subtypes are expressed in a highly restricted anatomic distribution in the central nervous system, suggesting that they play unique roles and are not functionally interchangeable.\(^6\) Outside of the central nervous system, Ca,3.x mRNA has been identified in endocrine tissue, testes, kidney, heart, coronary artery, and to a lesser extent in other tissues.\(^5\) Several studies have raised the possibility that T-type channels could be involved in regulating cell proliferation. However, the data are both cell-specific and highly contradictory.\(^7-9\) Given the potentially important role LVA channels could play in the regulation of cell cycle, using human pulmonary artery (PA) smooth muscle cells (SMCs) as a model system, we undertook this study to identify the molecular basis of the LVA current, and to test the hypothesis that Ca,3.x channels control smooth muscle cell proliferation.

Materials and Methods

Electrophysiology

Whole-cell currents were recorded using an EPC-9 amplifier with Pulse/PulseFit software (HEKA Elektronik) and filtered at 2.9 kHz. Voltage-dependent currents were corrected for linear leak and residual capacitance using an online P/n subtraction paradigm. Extracellular solution contained (in mmol/L) 10 CaCl\(_2\), 110 tetraethylammonium chloride, 10 CsCl, and 10 HEPES at pH 7.4. Pipette solution contained (in mmol/L) 130 N-methyl-D-glucamine, 10 EGTA, 5 BAPTA, 10 HEPES, 6 MgCl\(_2\), 4 CaCl\(_2\), and 2 Mg-ATP at pH 7.2. For some studies, nifedipine (1 \(\mu\)mol/L) or mibefradil (1 \(\mu\)mol/L) were added to the extracellular solution to inhibit Ca\(^{2+}\) channels.

RT/PCR

Primers were designed using Genbank sequences and the Perkin Elmer ABI Primer Express program both for human and rat genes.
Each primer was searched against BLAST to ensure that it did not match any known gene aside from that for which it was designed, including other family members. All primers were designed for an amplicon length of between 100 to 130 bp, which is optimal for quantitative RT-PCR, with primer melting temperatures of 59°C to 61°C and amplicon melting temperatures of 83°C to 85°C. Correct amplicon melting temperatures were verified using the ABI dissociation protocol, in which temperatures are increased from 60°C to 95°C in half-degree intervals, with quantity of double-stranded DNA checked at each temperature.

RNA was made using a Qiagen RNeasy mini kit. Rat lungs and brains were isolated from 5 adult male Sprague-Dawley rats and a pool of 5 adult male Sprague-Dawley rats and a pool of adult male Sprague-Dawley rats were obtained from Harlan Labs (Indianapolis, Ind) and euthanized with a lethal overdose of pentobarbital in concordance with procedures approved by the University of Colorado Health Sciences Center Laboratory Animal Care and Use Committee. Human lung and brain tissue was purchased from Clontech (Palo Alto, Calif). cDNA was made using Superscript II RT with oligo (dT)12 to 18 primers (both from Invitrogen). For screening gels, PCR was performed in a GeneAmp Sequence Detection System 5700 (Perkin Elmer) using 40 cycles of 95°C to 60°C PCR with a 10-minute 95°C initial soak. For quantitative PCR, the same equipment was used, but the fluorescent indicator Sybergreen was used to allow real-time light detection. Primer efficiency was determined and found to be 100% for all primers. Each measurement was made in triplicate and averaged, with 3 individual replicate experiments used for statistical analysis.

Preparation and Validation of Polyclonal Antibodies
Polyclonal anti-Ca$_{\text{v}}$3.1 and anti-Ca$_{\text{v}}$3.2 antibodies were prepared against peptides derived from the deduced T-type channel sequences $\alpha_{\text{1G}}$/Ca$_{\text{v}}$3.1: FVCQGEDTRNITNKSDCAEAS; $\alpha_{\text{1H}}$/Ca$_{\text{v}}$3.2: YYCEGPDRNISTKACRAAH and immunoaffinity-purified (Bethyl Laboratories). Antisera were immunoadsorbed to the common “DTRNI” peptide to eliminate potential cross-reactivity due to that epitope. Because the antibodies used to detect Ca$_{\text{v}}$3.1 and Ca$_{\text{v}}$3.2 had not been previously reported, we performed a series of experiments validating their use. Figure 1 demonstrates the specificity of the antibodies using heterologous expression in HEK cells. Brain was used as a positive control. These examples are representative of 5 replicates and show that the antibodies are selective and have roughly comparable sensitivity. Blocking peptide eliminated immunostaining, showing that the staining is epitope-specific.

Immunoblot
HEK cells were grown in 100 mm dishes to approximately 70% confluence. Cells were harvested for protein and Western blot run using standard methods. Membranes were probed with 1:200 anti-Ca$_{\text{v}}$3.1 or -Ca$_{\text{v}}$3.2, secondary was 1:1000 donkey anti-rabbit HRP. Similar studies were attempted using cultured human PA SMCs with human brain as a positive control. Although a band for Ca$_{\text{v}}$3.1 was detected, none was detected for Ca$_{\text{v}}$3.2 (data not shown).

Immunohistochemistry
Tissue blocks were obtained from the Department of Pathology at the University of Colorado Health Sciences Center under supervision of the institution’s Combined Institutional Review Board. Using standard procedures, sections were incubated in primary antibody overnight at 4°C at 1:250 dilution in PBS/4% goat serum/0.1% triton x-100 (PBS). Slides were rinsed and placed in either Alexa Fluor 488 or 594 (Molecular Probes) goat anti-rabbit secondary antibody at 1:1000 dilution for 1 hour at room temperature. Some slides were also stained for monoclonal anti–SMC $\alpha$-actin (Sigma Chemical) at 1:1000 dilution using the same primary and secondary incubation procedure. Immunohistochemistry followed identical protocols using 8-chamber Falcon Glass Culture slides (Beckton Dickinson). Images were captured with a scanning digital camera (Zeiss) and saved to a PC (Gateway) in TIFF format. For dark field microscopy, images were captured in either the red, green, or blue wavelength and merged in Photoshop CS (Adobe). A standard S-curve was applied to all dark field images to set contrast. Scale was calculated based on the magnification of objectives and transmission lenses and a scale bar placed digitally. For images so noted, a selection of the individual fluorescent channels was magnified digitally to 2 times original size to better show cellular localization.

Cell Culture
Normal human lung PA SMCs obtained from Clonetics/Biowhitetaker were cultured in SMC growth media (SmGM-2-bulletkit, Clonetics) with 5% fetal bovine serum (FBS; Gemini) and grown in humidified incubators (Forma Scientific) at 37°C in 5% CO$_2$, 16% O$_2$, balance N$_2$.

RNA Interference
siRNA target finder software (Ambion) was used to design 5 siRNA sequences for inhibition of human Ca$_{\text{v}}$3.1. Primers for chemical synthesis of siRNA were then constructed by Integrated Data Technologies, Inc and used as described in Ambion’s Silencer siRNA construction kit. Primer sequences are listed in the Table. siRNA directed against GAPDH and scrambled siRNA (negative) controls were synthesized using standard templates (Ambion). Then, 0.25 µL of 20 µmol/L stock siRNA was transferred into HEK cells stably expressing Ca$_{\text{v}}$3.1, or primary cultures of human PA SMCs using Lipofectamine Plus (Invitrogen). The function of Ca$_{\text{v}}$3.1
siRNA was validated using Western blotting for protein of cell lysate 24 to 48 hours after addition of siRNA in triplicate, using standard techniques. Figure 2A shows a representative Western blot demonstrating that Cav3.1 siRNA decreased Cav3.1 protein in HEK cells stably expressing Cav3.1. Consistent with the Western blot data for protein, Cav3.1 siRNA reduced Cav3.1 current density by approximately 50%. It is noteworthy that stably transfected HEK cells expressed T-type current in the 50 to 100 pA range, whereas endogenous PA SMC T-type current was in the 5 to 10 pA range. Therefore, one would expect that suppression of endogenous T-type current would be greater than the 50% reduction seen in these experiments, which is consistent with the greater degree of inhibition of Cav3.1 protein expression detected by Western blot in PA SMCs (Figure 5).

Cell Counts and Flow Cytometry
PA SMCs, passage 2 to 6, were plated at 3750 cells/cm² into 24-well plates and allowed to sit overnight in growth media. Cells were placed into the appropriate treatment group, and cell count and cycle data were collected on days 1, 3, 5, and 7. All treatment groups contained growth media with 5% FBS with the exception of the nonproliferation control, which contained 0.1% FBS. Mean and SEM for six replicates were calculated for cell counts. Cell viability was confirmed by trypan blue exclusion. Cell cycle was determined after 24 hours of treatment by flow cytometry in propidium iodide–stained human PA SMCs as previously described.

Statistical Methods
Group means were compared using either Students t test or ANOVA with Fisher post hoc test as appropriate. Data are presented as mean±SEM with P<0.05 accepted as significant.

Results
Electrophysiology
To confirm functional expression of LVA Ca²⁺ channels in PA SMCs, whole-cell patch clamp was used. Resting Em of PA SMCs was −48±2 mV under the experimental conditions. Figure 3 demonstrates macroscopic inward currents. A small, rapidly inactivating inward current with peak current density at −40mV and a larger, sustained inward current with peak current density at +10mV were apparent when the holding potential was −90mV. Nifedipine (1 μmol/L) eliminated the sustained, but not the transient inward current. Current-voltage relationships compiled when depolarizing from a holding potential of −90mV demonstrated the two components of the inward current, with a small peak at −40mV and a larger peak at +10mV. Treatment with nifedipine eliminated the typical L-type current-voltage relationship with peak inward current at +10mV, revealing the smaller transient inward current with peak at −40mV, typical of Cav3.1.

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CCTGTGTC tag on each sequence is used for the chemical synthesis protocol.

Figure 2. Effect of Cav3.1 siRNA on T-type channel protein expression and current density in stably transfected HEK cells. A, Western blots showing the effect of a mixture of all five Cav3.1 siRNAs (si3.1) on Cav3.1 protein expression in HEK cells stably transfected with Cav3.1, or Cav3.2. Cells transfected with either scrambled siRNA (S) or mock transfected with lipid alone (M) serve as controls. Right blot shows that Cav3.1 siRNA did not suppress Cav3.2 in cells stably expressing Cav3.2. B, Macroscopic currents from HEK cells transfected with Cav3.1 cDNA (left) then treated with Cav3.1 siRNA (center). I-V curves from the entire groups show that siRNA suppressed maximum inward current without affecting biophysical properties. C, Group means for maximum current density measured at −40mV in stably transfected cells demonstrating the ability of Cav3.1 siRNA to diminish Cav3.1 current.
of T-type currents, whereas mibefradil (1 μmol/L) inhibited both components of the inward current. These inward currents are consistent with the presence of both HVA, L-type, and LVA, T-type, Ca2+ channels in PA SMCs.

RT/PCR Analysis of T-Type Channel Gene Expression in PA SMCs and Lung
Figure 4A shows a representative gel demonstrating the expression pattern of α1 subunits in cultured human PA SMCs. As expected, Ca1.2, encoding the HVA L-type Ca2+ channel, was detected in PA SMCs. Ca3.1 and Ca3.2 were also detected, whereas Ca3.3 was not readily appreciated. Using real-time PCR, the relative expression levels of the transcripts was estimated. Figure 4B shows the number of PCR cycles required for detection above that required for β-actin in the same samples. The results show that Ca3.1 was the most abundant of the 3 Ca3 transcripts and Ca3.3 least abundant, with the relative expression levels for Ca3.1 approximately 4-fold greater than Ca3.2 and 20-fold greater than Ca3.3 (P<0.01 between all groups). In both rat and human lung, Ca3.1 and Ca3.2 could be detected, whereas Ca3.3 was either absent, or much less abundant.

Cellular Localization of Ca3.1
Figure 5 shows triple labeling for smooth muscle α-actin (red), Ca3.1 (green), and nuclei (DAPI, blue) in lung tissue. Colocalization of red and green fluorescence could be readily detected in cells in the media of both large and small pulmonary arteries. A similar pattern was seen in bundles of cells in the wall of small airways. Expression of Ca3.1 was

Figure 3. Whole-cell recordings from PA SMCs. Ca2+ (10 mmol/L) was used as a charge carrier. A, Traces demonstrate T- and L-type Ca2+ current elicited at −40 and +10 mV when membrane potential was held at −90 mV. Note the T-type Ca2+ current inactivates within 50 ms. B, Averaged current-voltage relationship of peak currents obtained from 6 PA SMCs when holding at −90mV. C and D, Effect of nifedipine (1 μmol/L, “N”), which eliminated the sustained HVA component seen at more positive membrane potentials. Effect of nifedipine was reversible (tracing +10 –N is an example of maximum inward current after washout of nifedipine). Mibefradil (1 μmol/L) eliminated all inward currents (averaged current-voltage relationship from 3 replicates shown in open circles in D; error bars were omitted for clarity). The absolute magnitude of inward currents was small, with the maximum current for the LVA component being 5±2 pA, whereas it was 35±11 pA for the HVA component (P<0.05).
detected in both large and small pulmonary arteries, whereas Cav3.2 was not detected. Figure 5 also shows immunocytochemistry of cultured PA SMCs, demonstrating expression of Cav3.1, but not Cav3.2.

Effects of siRNA on Proliferation
Figure 6 shows the effects of siRNA on Cav3.1 expression and proliferation. Figure 6A through 6C demonstrates the high efficiency and efficacy of lipid-mediated delivery of siRNA to human PA SMCs, using fluorescently labeled, prevalidated GAPDH-targeted siRNA. Figure 6D shows the effect of siRNA directed against Cav3.1 on protein expression in PA SMCs. This is representative of 3 replicates, all showing that expression of Cav3.1 protein was partially suppressed by the siRNA sequence targeting bases 432 to 452 and reduced below the level of detection by Western blot by the remaining 4 siRNA sequences. The effect of Cav3.1 siRNA on proliferation is shown in Figure 5E. The 4 siRNAs that blocked Cav3.1 protein expression most effectively also inhibited proliferation, whereas control conditions had only a modest nonspecific effect. In addition, the siRNA sequence that had an incomplete effect on Cav3.1 protein also had an intermediate effect on proliferation.

Effect of Pharmacological VOCC Blockade on Proliferation and Cell Cycle
Figure 7A shows the effect of pharmacological VOCC blockade on proliferation. Five percent serum and 0.1% serum were used as positive and negative controls, respectively. The combined L- and T-type Ca2+ channel blocker mibebradil completely blocked the ability of 5% serum to induce proliferation, whereas the L-type Ca2+ channel blocker diltiazem had no effect when used at a concentration of 10 μmol/L (3 times the EC50) and only a modest effect when used at 5 times that concentration (which exceeds the concentration that is selective for L-type channels). Figure 7B shows a summary of FACS analysis of cell cycle, demonstrating that, when compared with diltiazem-treated cells, those treated with either low extracellular Ca2+ or mibebradil showed a much smaller proportion of cells entering S and G2/M, and a much higher proportion arrested in G0/G1. These examples are representative of 3 experiments. For comparing cells arrested in G0/G1 between groups, P=NS between mibebradil and 0.1% serum, P<0.05 between mibebradil and diltiazem, and P=NS between 10 μmol/L diltiazem and 5% serum. No evidence of cell necrosis or apoptosis was detected either grossly or by FACS (as DNA fragmentation).

Discussion
Electrophysiologically, both L- and T-type VOCCs have been identified in vascular SMCs. In the pulmonary circulation, the role of L-type VOCCs in control of vascular tone is well established, and pharmacological L-type blockade results in marked attenuation of hypoxic pulmonary vasoconstriction. In contrast, the role of T-type channels in the regulation of PA tone and structure is less well established. In support of the
hypothesis that T-type VOCCs are expressed in the pulmonary circulation, we previously reported that T-type blockade was necessary to eliminate abnormal vasoconstriction in chronically hypoxic rat lungs treated with nitric oxide synthase blockers. We therefore undertook the present study to identify the gene(s) encoding T-type VOCCs and establish their functional significance in PA SMCs.

To confirm the physiological presence of LVA channels, we performed whole-cell patch clamp on early passage, cultured PA SMCs, detecting both LVA and HVA Ca2+ currents. Using RT/PCR, we determined that cultured human PA SMCs expressed mRNA for the T-type Cav3.1 and Cav3.2, and to a much smaller extent Cav3.3. Immunostaining demonstrated expression of Cav3.1 but not Cav3.2, suggesting that only the mRNA for Cav3.1 was being transcribed into detectable amounts of protein. Although we do not have an explanation for the apparent lack of translation of Cav3.2 mRNA into protein, several recent studies implicate posttranslational control of protein expression based on translational efficiency. It is also possible that the Cav3.2 antibody was unable to detect small amounts of endogenously expressed protein.

Consistent with the in vitro results, in human lung tissue, mRNA for Cav3.1 and Cav3.2, but not Cav3.3, was detected.

To identify the cell types in lung expressing Cav3.1, we used dual staining for smooth muscle α-actin and Cav3.1 and localized Cav3.1 expression in lung predominantly to SMCs of both large and small pulmonary arteries. In addition, SMCs in small airways expressed Cav3.1, suggesting Cav3.1 may encode the T-type Ca2+ current previously reported in bronchial SMCs. As with the cell culture studies, Cav3.2 protein was not detected in lung tissue.

Our prior studies suggested that T-type channels were important in the remodeled, chronically hypertensive pulmonary circulation, suggesting they could become more prominent in proliferating PA SMCs, possibly playing a role in controlling PA SMC proliferation. In support of that hypothesis, in rat systemic vascular SMCs, the percentage of cells with an electrophysiologically detectable T-type current increased from nondetectable during G0 to 37% and 90% during G1 and S-phase, respectively. Consistent with a role for T-type channels in vascular SMC proliferation, mibefradil prevented neointima formation in a carotid balloon injury model in rats. However, when the hypothesis that T-type channels regulate cell proliferation was tested by overexpressing either Cav3.1 or Cav3.2 in HEK293 (human embryonic kidney) cells, increased Ca2+ influx but no proliferative advantage, as assessed by FACS analysis of cell cycle, was
seen. More recently, several studies have implicated T-type channels in the proliferation of tumor cells. Thus, the published literature is conflicted on the role of T-type Ca\textsuperscript{2+} channels in control of proliferation. These conflicting studies could be reconciled, however, if the temporal control of endogenous Cav3.x gene expression, rather than constitutive, sustained overexpression, was necessary in SMCs. We addressed that possibility by testing the ability of siRNA directed against Cav3.1 to inhibit PA SMC proliferation. The ability of Cav3.1 siRNA to block Cav3.1 protein expression and macroscopic currents was verified in heterologous expression studies in HEK cells. In PA SMCs, Cav3.1-targeted siRNA blocked both channel protein synthesis and proliferation. The nonspecific effect of lipofection (sham group) and GAPDH-targeted siRNA were modest and likely due to time- and dose-dependent toxicity of lipid-nucleotide complexes. In contrast, 4 of 5 Cav3.1-targeted siRNAs reduced proliferation by 92% to 98%. The one exception was the sequence that targeted bases 432 to 452. That siRNA sequence was less effective at reducing Cav3.1 protein, and, consistent with less effective protein knockdown, reduced proliferation by an intermediate value of 65%. Although we did not specifically verify that Cav3.1 siRNA did not affect expression of other ion channels, it is highly unlikely that concordant results from 5 distinct siRNA sequences targeted against Cav3.1 were the result of unanticipated sequence-
specific suppression of other ion channel genes. Consistent with a role for Cav3.1 in controlling cell cycle, similar effects on proliferation were recently reported using antisense oligonucleotides directed against Cav3.1 in tumor cells.23 Thus, it appears that Cav3.1 expression is necessary for serum-stimulated proliferation of PA SMCs.

Cell proliferation within normally quiescent vascular smooth muscle cells requires first entry and then progression through the cell cycle toward mitosis. The L-type Ca$^{2+}$ channel blocker diltiazem had little effect on cell proliferation, whereas the T-type blocker mibebradil completely inhibited proliferation. Consistent with this result mibebradil, but not diltiazem, increased the number of cells arrested in G0/G1 as determined by flow cytometry. These findings, along with lack of evidence of cell necrosis or apoptosis, lend further support to the hypothesis that T-type Ca$^{2+}$ channel activation is necessary for serum-stimulated PA smooth muscle cell cycle progression and proliferation.

While our studies suggest that T-type Ca$^{2+}$ channel activation is necessary for proliferation, it is likely that complex signaling processes involving multiple ion channels, including Ca$^{2+}$-activated K$^+$ (KCa) channels and store-operated Ca$^{2+}$ entry, are required for the full mitogenic response. Some insight into complex vascular signaling involving T-type channels was reported in a recent study of Ca$^{2+}$-null mice.24 Although the pulmonary vascular phenotype was not studied, they found significant myocardial fibrosis, associated with coronary artery spasm and impaired NO-mediated coronary artery relaxation. This suggests that in mouse coronary artery, T-type channels, encoded by Cav3.2, may be involved in regulating membrane potential, possibly by activating KCa channels. In a study of rat PA SMCs, Ca$^{2+}$ entry via store-operated channels (SOCs) encoded by Trp6 was required for PDGF-stimulated proliferation, suggesting a requirement for SOCs as well.25 These observations could be explained by a model in which Ca$^{2+}$ entry via T-type channels provides “capacitative calcium entry” in endothelial cells, as was recently described in tumor cells.22 However, one must be cautious in integrating the results of these studies, as it is highly likely that the role of T-type -1 subunits in regulating membrane potential and cell proliferation is both cell-type and species specific. Thus, studies in each cell type will be required to ascertain the molecular identity of T-type channels and establish their role in controlling proliferation.

In conclusion, these studies establish the existence of the T-type Ca$^{2+}$ channel -1 subunit Cav3.1 in human pulmonary artery SMCs both in vitro and in vivo. RNA interference and pharmacological inhibitor studies provide direct evidence that T-type Ca$^{2+}$ channels are important regulators of proliferation in pulmonary artery smooth muscle cells. Future studies will be required to determine the mechanisms through which T-type Ca$^{2+}$ channels regulate cell cycle progression and the relevance of these studies to control of cell cycle and proliferation in other cell types.

Limitations of the Study

We were unable to validate the specificity of Cav3.1 siRNA on endogenous Cav3.1 expression and function in PA SMCs due to the difficulty in maintaining adequate patch electrode seals in siRNA/lipid complex–treated cells. Therefore, we relied on indirect evidence from heterologous expression studies that the siRNA strategy we used was specific for Cav3.1 and did not affect Cav3.2 or other signaling pathways. A second limitation is the inability to be certain that the Cav3.2 antibody, although apparently specific for Cav3.2 and sensitive enough to detect protein expression in brain and heterologous expression studies, was sensitive enough to detect endogenous Cav3.2 protein in vascular tissue. Therefore, although our studies strongly support a relationship between Cav3.1 expression and control of PA SMC proliferation, they do not completely rule out a contribution of Cav3.2 to control of PA SMC proliferation, nor do they rule out a role for Cav3.2 in endothelial cell function and paracrine regulation of SMC function, in vivo.

Acknowledgments

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References

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