A naturally occurring truncated Cav1.2 α₁-subunit inhibits Ca^{2+} current in A7r5 cells

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Cox RH, Fromme SJ. A naturally occurring truncated Cav1.2 α₁-subunit inhibits Ca^{2+} current in A7r5 cells. Am J Physiol Cell Physiol 305: C896–C905, 2013. First published August 7, 2013; doi:10.1152/ajpcell.00217.2013.—Alternative splicing of the voltage-gated Ca^{2+} (CaV) α₁-subunit adds to the functional diversity of CaV channels. A variant with a 73-nt deletion in exon 15 of the Cav1.2 α₁-subunit (Cav1.2Δ73) produced by alternative splicing that predicts a truncated protein has been described, but its function, if any, is unknown. We sought to determine if, by analogy to other truncated CaV α₁-subunits, Cav1.2Δ73 acts as an inhibitor of wild-type Cav1.2 currents. HEK-293 cells were transfected with Cav1.2Δ73 in a pIRE vector with CD8 or in pcDNA3.1 with a V5/His COOH-terminal tag plus β2 and αδ1 accessory subunits and pEGFP. Production of Cav1.2Δ73 protein was confirmed by Western blotting and immunofluorescence. Voltage-clamp studies revealed the absence of functional channels in transfected cells. In contrast, cells transfected with full-length Cav1.2 plus accessory subunits and pEGFP exhibited robust Ca^{2+} currents. A7r5 cells exhibited endogenous Cav1.2-based currents that were greatly reduced (>80%) without a change in voltage-dependent activation when transfected with Cav1.2Δ73-IRES-CD8 compared with empty vector or pIRE-CD8 controls. Transfection of A7r5 cells with an analogous Cav2.3Δ73-IRES-CD8 had no effect on Ca^{2+} currents. Immunofluorescence showed intracellular, but not plasma membrane, localization of Cav1.2Δ73-V5/His, as well as colocalization with an endoplasmic reticulum marker, ER Organelle Lights. Expression of Cav1.2Δ73 α₁-subunits in A7r5 cells inhibits endogenous Cav1.2 currents. The fact that this variant arises naturally by alternative splicing raises the possibility that it may represent a physiological mechanism to modulate Cav1.2 functional activity.

Cav1.2 subunits; alternative splicing; truncated Cav1.2 transcripts; A7r5 cells; endoplasmic reticulum

VOLTAGE-GATED Ca^{2+} channels (CaV) play an important role in the function of many cell types, including muscle, where they provide activator Ca^{2+} for excitation-contraction coupling (2, 23). In arterial smooth muscle cells (ASMCs), a voltage window exists within which CaV provide sustained Ca^{2+} influx (32) and sustained increases in intracellular Ca^{2+} concentration (9) in the physiological range of membrane potential. The relative contribution of CaV to Ca^{2+} influx in ASMCs varies with anatomic location and vessel size, but in resistance arteries, Cav are thought to represent the primary Ca^{2+} influx pathway (23). In addition, their relative contribution to Ca^{2+} influx is enhanced in peripheral vascular diseases, including hypertension (7), where L-type CaV blockers represent an important therapeutic drug class (7, 34).

Ten genes code for distinct CaV α₁ pore-forming subunits that are assembled with up to four accessory subunits (7). Although ASMCs from some sites have been reported to functionally express channels formed from multiple Cav α₁-subunit families (11, 21), it appears that the Cav1.2 α₁-subunit is expressed in all ASMCs and may represent the dominant form (7). In consonance, conditional smooth muscle-specific Cav1.2 α₁-subunit deletion in mice produced a substantial reduction in blood pressure, blunted blood pressure responses to vasoconstrictor agents, eliminated myogenic responses in tibial arteries, and greatly reduced CaV currents in isolated tibial artery myocytes (20). Also, direct measurements of Ca^{2+} current in freshly isolated myocytes from rat small mesenteric arteries (SMAs) showed the presence of only Cav1.2 α₁-subunits on the basis of the observation that 100% of the current was inhibited by dihydropyridines (DHPs) (17).

It is well recognized that alternative splicing of pre-mRNA adds to the functional diversity of proteins, including CaV (35). In the course of analyzing splice variants of Cav1.2 α₁-subunits expressed in SMA myocytes, we found a transcript with a 73-nt deletion (Cav1.2Δ73) in multiple preparations. This transcript predicts a protein that is truncated in the P-S6 region of domain II (exon 15) and was originally described in human fibroblasts (33). On the basis of studies showing that some truncated CaV α₁-subunits act as dominant-negative regulators of full-length channel function (13, 19, 28), we hypothesized such a role for Cav1.2Δ73 subunits. We tested this hypothesis by expressing Cav1.2Δ73 in human embryonic kidney (HEK)-293 and A7r5 cells. The latter express endogenous Cav1.2 α₁-subunits and functional CaV channels, while the former do not. We found that expression of Cav1.2Δ73 leads to production of truncated protein in both cell lines, does not lead to formation of functional channels when expressed with CaV β₂- and αδ1-subunits in HEK cells, reduces endogenous Cav1.2 currents in A7r5 cells, and is retained in the endoplasmic reticulum in A7r5 cells.

METHODS

Animals and Tissues

Tissues were removed from 15- to 18-wk-old male Wistar rats following euthanasia with CO₂. Animals were handled according to the principles described in the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The protocol involving the use of laboratory animals was approved by the Lankenau Institute for Medical Research Animal Care and Use Committee.

RNA Isolation and RT-PCR

The medium was removed from cultured cells, and the cells were washed with cold PBS; then 0.5 ml (for a 6-well plate or 60-mm dish)
or 1 ml (for a 100-mm dish) of TRI Reagent (RiboPure kit, Ambion) was added for 30 s. Cells were removed in TRI Reagent by scraping, transferred to a microcentrifuge tube, and incubated at 4°C for 15 min on a nutator, and total RNA was isolated following the manufacturer’s protocol. ASMCs were enzymatically isolated from third- to fifth-order SMAs, as described elsewhere (6). Briefly, mesenteric artery networks were isolated and placed in a Ca2+-free solution of the following composition (mmol/l): 120 NaCl, 25 NaHCO3, 4.2 KCl, 0.6 KH2PO4, 1.2 MgCl2, and 11 glucose (pH 7.4, at room temperature). Air was bubbled through the lumen for 5 min to remove endothelial cells. SMAs treated in this manner do not respond to endothelium-mediated relaxations by acetylcholine but do respond to nitroprusside. Arteries were then opened longitudinally with microscissors and incubated for 15 min at 37°C in Ca2+-free buffer. The tissues were transferred to fresh solution containing papain (0.3 mg/ml; Worthington Biochemical, Lakewood, NJ), bovine serum albumin (1 mg/ml; USB, Cleveland, OH), and DTT (1 mg/ml; Sigma Chemical, St. Louis, MO) and incubated for 15 min at 37°C. The tissue was washed again with Ca2+ (70% type F and 30% type H; Sigma Chemical) in the same solution and gently triturated in 1 ml of fresh solution at room temperature. Released cells were separated from tissue fragments by filtration through a 40-mm nylon mesh cell strainer (Fisher Scientific, Pittsburgh, PA), pelleted by centrifugation at 1,000 g for 10 min at 4°C, and resuspended in 0.5 ml of TRI Reagent. RNA was isolated according to the manufacturer’s protocol.

Total RNA was also obtained from intact tissues (rat heart and human sources) by homogenization on ice (Polytron, Fisher Scientific) for 15 min at 37°C. The tissue was washed again with Ca2+-free solution and gently triturated in 1 ml of fresh solution at room temperature. Released cells were separated from tissue fragments by filtration through a 40-μm nylon mesh cell strainer (Fisher Scientific, Pittsburgh, PA), pelleted by centrifugation at 1,000 g for 10 min at 4°C, and resuspended in 0.5 ml of TRI Reagent. RNA was isolated according to the manufacturer’s protocol.

An artificial Cav2.3Δ73-IREs-CDS8 construct was created by amplification of amino acids 1–654 of Cav2.3 (NM_019294) with Advantage HF2 polymerase, with cDNA synthesized from rat brain RNA used as template and the following primers: AAG CTA GCC ACC ATG GCT CGC TTC GGA GAG (forward) and ACC AGT CAC AAC AGT GAG (reverse). The PCR fragment was gel-purified and cloned into the pIREs-CDS8 construct as a NheI/MluI insert.

Plasmid constructs for Cav1.2 α1, β2, and αδβ1 in pcDNA3 were kindly provided by Dr. Richard Swanson. All constructs were sequenced completely on both strands to confirm their accuracy. The Cav1.2 construct, including exons 1b, 8, 9*, 15 complete, 21, 32, and 33, but excluding exon 45, was similar to that of the smooth muscle variant (4, 35).

**Sequencing**

PCR products were gel-purified, and A overhangs were added (A-Addition kit, Qiagen). The products were then TA-cloned into pCRII TOPO (Invitrogen) and used to transform TOP10F’ cells (Life Technologies). Cells were layered on LB-ampicillin agar plates with X-gal/isonicotinamide thiogalactoside (KD Medical, Columbia, MD) for blue-white selection and incubated at 37°C overnight. Colonies were selected, cultured overnight in LB-ampicillin medium, and used for isolation of plasmid DNA (Miniprep, Qiagen), which was sequenced on both strands at the Kimmel Cancer Center of Thomas Jefferson University.

**Cell Culture and Transfection**

HEK-293 and A7r5 cells (American Type Culture Collection, Manassas, VA) were maintained in MEM and DMEM (American Type Culture Collection), respectively, with 10% fetal bovine serum plus 2 mM L-glutamine (Life Technologies) without antibiotics at 37°C with 5% CO2 in air. Cultured cells were consistently negative for Mycoplasma contamination (RADIL, Columbia, MO). Cells were transfected with Cav constructs using FuGENE HD according to the manufacturer’s protocol (Roche Diagnostics, Indianapolis, IN). For ER localization, cells were first transfected with Cav1.2Δ73-V5/His plus β2 and αδβ1 constructs using FuGENE HD and selected with G418 after 7 days. Selected HEK or A7r5 cells were transfected with ER Organellene Lights (Invitrogen) according to the manufacturer’s recommendations and analyzed 24 h later.

**Western Blots**

Cells cultured on 100-mm plates were washed twice with 3 ml of cold PBS and then with 1 ml of RIPA reagent (Sigma Chemical) with protease (Complete Mini, Roche Diagnostics), and phosphatase inhibitors (Halt, Pierce Biotechnology, Rockford, IL) were added for 2 min at 4°C. The PBS had the following composition (mmol/l): 137 NaCl, 2.7 KCl, 10 Na2HPO4, and 2 K2HPO4 (pH 7.4). Cells were scraped from the plates in the RIPA buffer, transferred to a microcentrifuge tube, incubated on a nutator for 15 min at 4°C, and centrifuged at 10,000 rpm (9,800 g) for 10 min at 4°C. The supernatant was removed and analyzed for protein content (DC Protein Assay, Bio-Rad, Hercules, CA), with albumin used as a standard. Aliquots of proteins were combined with 4× Laemmli sample buffer heated for 5 min at 70°C or 97°C and size-fractioned by electrophoresis using 7% Tris-acetate or 10% Tris-glycine gels (Invitrogen) (6). Proteins were transferred to polyvinylidene difluoride membranes (Immobilon-P, Millipore, Billerica, MA) and blocked for 1 h at room temperature with 5% dry milk in Tris-buffered saline (TBS) with 0.1% Tween 20 (6). The TBS had the following composition (mmol/l): 137 NaCl, 2.7 KCl, and 24.7 Tris base (pH 7.4). Membranes were incubated with primary antibody overnight at 4°C, washed, and incubated with horseradish peroxidase-conjugated secondary antibody in TBS with 0.1% Tween 20 (Jackson ImmunoResearch, West Grove, PA). After washing, membranes were incubated with horseradish peroxidase-conjugated secondary antibody in TBS with 0.1% Tween 20 (Jackson ImmunoResearch, West Grove, PA). After washing, membranes were incubated with horseradish peroxidase-conjugated secondary antibody in TBS with 0.1% Tween 20 (Jackson ImmunoResearch, West Grove, PA). After washing, membranes were incubated with horseradish peroxidase-conjugated secondary antibody in TBS with 0.1% Tween 20 (Jackson ImmunoResearch, West Grove, PA).
PA) for 1 h at room temperature. Immunoreactivity was detected by chemiluminescence (ECL or ECL+, Amersham Biosciences, Piscataway, NJ), and signals were recorded on film (Hybblot, Denville Scientific, Metuchen, NJ).

Cell Surface Protein Biotinylation

Surface proteins of cells on 60-mm plates were biotinylated using a cross-linking reagent (EZ-Link Sulfo-NHS-S-S-Biotin, Pierce Biotechnology) (6). Cells were washed three times with ice-cold PBS, and 1 mg/ml of biotinylation reagent in 2.5 ml of biotinylation buffer (in mmol/l: 150 NaCl, 2 CaCl₂, and 10 triethanolamine) or buffer alone was added for 30 min at room temperature. After the cells were washed and quenched, whole cell protein lysates were isolated using Triton lysis buffer (150 mmol/l NaCl, 1.5 mmol/l MgCl₂, 20 mmol/l HEPES, 1% Triton X-100, and 10% glycerol, pH 7.5) with protease and phosphatase inhibitors. Biotinylated proteins were recovered from the cell lysates with streptavidin-coated agarose beads (Sigma Chemical, Rockford, IL). Biotinylated and nonbiotinylated proteins plus precleared whole cell lysates were prepared for Western blotting, as described above.

Cellular Immunofluorescence

Cells were released with trypsin, plated on glass slides, allowed to adhere for 30 min, and fixed with cold 4% paraformaldehyde. The slides were washed and incubated in blocking buffer consisting of 10% dry milk in PBS with 0.1% Triton X-100 for 1 h at room temperature. Cells were then incubated with primary antibody in blocking buffer overnight at 4°C. Cells were subsequently washed three times with cold PBS and incubated with Cy3-conjugated secondary antibody in blocking buffer at room temperature for 60 min in the absence of light. Cells were again washed three times with cold PBS and briefly air-dried, and Prolong Gold (Invitrogen) mounting medium with 4,6-diamidino-2-phenylindole was added to the slides. The slides were covered with glass coverslips and viewed using a Zeiss Axioscope microscope with a Plan Neofluar 40×/0.75 oil immersion objective for Cy3 fluorescence. Images were captured with an Axioscan charge-coupled device camera using AxioVision software (Zeiss).

Voltage-Clamp Studies

Electrophysiology. HEK or A7r5 cells on coverslips were placed in a chamber and, where appropriate, incubated with 2 μl of CD8 Dynabeads (Invitrogen) in 1 ml of external voltage-clamp solution with gentle shaking for 10 min. The chamber was then placed on the stage of an inverted microscope (Diaphot, Nikon Instruments, Tokyo, with gentle shaking for 10 min. The chamber was then placed on the stage of an inverted microscope (Diaphot, Nikon Instruments, Tokyo, Japan) and perfused by gravity at 1 ml/min with external voltage-clamp solution containing (mmol/l) 140 NaCl, 5 KCl, 1 MgCl₂, 2 CaCl₂, 10 HEPES, and 10 glucose, with pH adjusted to 7.4 by addition of NaOH. Ca²⁺ currents were recorded in the whole cell configuration at room temperature (22°C) with an Axopatch 200B amplifier (Molecular Devices, Foster City, CA) using micropipettes fabricated from borosilicate glass (WPI, Sarasota, FL) (22). Pipettes had resistances of 2–3 MΩ when filled with internal solution containing (mmol/l) 100 CsCl, 20 tetraethylammonium chloride, 5 NaCl, 1 MgCl₂, 5 Na₂ATP, 20 HEPES, 10 BAPTA, 0.01 cAMP, and 0.1 NaGTP, with pH adjusted to 7.3 with addition of CsOH. Pipette offset was compensated with the tip in the bath solution, and stray capacitance was minimized after high-resistance seal formation. After cell break-in, membrane capacitance and series resistance were compensated optimally. Series resistance prediction and correction were adjusted to >85%, leaving an uncompensated series resistance of <1 MΩ, and voltage errors were estimated to be <5 mV. Data from cells with voltage errors >5 mV due to uncompensated series resistance were excluded. Currents and voltage protocols were sampled at 10 kHz for offline analysis using Clampfit (version 10.2, Molecular Devices, Sunnyvale, CA).

Cell break-in was performed at a holding potential of −60 mV, and membrane potential was varied using a 100-ms ramp protocol from −60 to +40 mV at 15-s intervals until Ca²⁺ current stabilized (~3–5 min). Voltage-clamp protocols were then performed from a holding potential of −80 mV with 10-mV, 100-ms steps to test potentials from −60 to +40 mV at 20-s intervals. Leak currents were compensated using P/N leak subtraction. Experimental protocols were generated, and currents were acquired and analyzed using pCLAMP software (version 10.2, Molecular Devices). The external solution contained 2 mM Ca²⁺ unless otherwise noted. In most experiments, 500 μM amiloride was added to the external solution to inhibit endogenous T-type Ca²⁺ currents in A7r5 cells (22).

Statistics

Statistical analysis was performed using SigmaPlot (version 11.0, Systat Software, San Jose, CA). Comparisons of current-voltage data between groups were made with one- or two-way ANOVA. When the ANOVA produced a significant P value, comparisons were made using Bonferroni’s test for paired data or Dunnett’s test for multiple comparisons. P < 0.05 was considered statistically significant. Values are means ± SE.

RESULTS

Cav1.2Δ73 Transcripts Are Widely Distributed

A portion of domain II of the Cav1.2 α₁-subunit (exons 11–17) was amplified by conventional RT-PCR with a high-fidelity polymerase (Advantage HF2) and total RNA prepared from enzymatically dispersed rat SMA myocytes. Sequencing of the PCR product showed a 73-nt deletion in the P-S6 region of domain II (exon 15) of multiple clones (Fig. 1A). To ensure that this finding was not an artifact, we repeated this analysis

![Ethidium bromide-stained agarose gel of PCR products](http://ajpcell.physiology.org/)

Fig. 1. Cav1.2Δ73 transcripts are present in rat and human cardiovascular tissues, as well as brain. A: partial sequence alignment of the P-S6 region of domain II small mesenteric artery (SMA) voltage-activated Ca²⁺ (Caᵥ) channel (Cav1.2) clones shows variants with and without the 73-nt deletion (Δ73). FL, full-length. B: predicted amino acid sequences of the 2 variants show addition of 2 new amino acids (underlined) followed by a stop codon after the frame change. C: ethidium bromide-stained agarose gel of PCR products amplified from a portion of domain II shows 2 bands in rat left ventricle (LV), SMA, and brain (B) cDNA. D: ethidium bromide-stained agarose gel of PCR products for human LV, radial artery (RA), saphenous vein (SV), and brain shows similar results.

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Cells were released with trypsin, plated on glass slides, allowed to adhere for 30 min, and fixed with cold 4% paraformaldehyde. The slides were washed and incubated in blocking buffer consisting of 10% dry milk in PBS with 0.1% Triton X-100 for 1 h at room temperature. Cells were then incubated with primary antibody in blocking buffer overnight at 4°C. Cells were subsequently washed three times with cold PBS and incubated with Cy3-conjugated secondary antibody in blocking buffer at room temperature for 60 min in the absence of light. Cells were again washed three times with cold PBS and briefly air-dried, and Prolong Gold (Invitrogen) mounting medium with 4,6-diamidino-2-phenylindole was added to the slides. The slides were covered with glass coverslips and viewed using a Zeiss Axioscope microscope with a Plan Neofluar 40×/0.75 oil immersion objective for Cy3 fluorescence. Images were captured with an Axioscan charge-coupled device camera using AxioVision software (Zeiss).

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using RNA from multiple animals (n = 8) and found that ~26% (16 of 62) of clones had the 73-nt deletion. This deletion predicts a frame change with the addition of two unique amino acids in the translated protein followed by a stop codon (Fig. 1B).

To determine if this transcript was unique to rat SMA, we repeated this analysis using total RNA prepared from the left ventricular free wall and the brain of rat. As shown in Fig. 1C, we found two PCR products in the P-S6 region of domain II in all samples, and sequencing confirmed that the smaller product had the 73-nt deletion (Cav1.2Δ73). This analysis was repeated using cDNA prepared from total RNA isolated from discarded surgical samples of human left ventricle, radial artery, and saphenous vein, along with commercial human brain RNA (Clontech). All tissues showed the presence of two variants (Fig. 1D), with the smaller variant confirmed to be Cav1.2Δ73 by sequencing.

Cav1.2Δ73 Protein Is Synthesized

These results suggest that Cav1.2 α1 transcripts with a 73-nt deletion are present in multiple cardiovascular tissues, as well as in brain, of rat and human. To demonstrate that this transcript can direct the production of protein, we transfected HEK-293 cells with the following constructs: 1) Cav1.2Δ73 in a pIREs vector with CD8 and 2) Cav1.2Δ73 with a COOH-terminal V5/ his tag. Decoration Cav1.2Δ73-IREs-CD8-transfected HEK cells with CD8-labeled Dynabeads could be demonstrated 24 h after transfection by light microscopy, as could expression of V5 in cells transfected with Cav1.2Δ73-V5/ his by immunofluorescence (Fig. 2A). Protein lysates from HEK cells transfected with the two Cav1.2Δ73 constructs, as well as with pIREs-CD8 without Cav1.2Δ73, and cells treated with FuGENE HD alone were analyzed by Western blotting. An anti-Cav1.2 antibody to exon 1b (catalog no. ACC-022, Alomone Labs, Jerusalem, Israel) showed protein bands at ~70 kDa only in lysates from HEK cells transfected with Cav1.2Δ73 constructs (Fig. 2B). A protein band of the same size was detected using an anti-V5 antibody (Sigma Chemical) in lysates from cells transfected with the Cav1.2Δ73-V5/ his construct only (Fig. 2B). Voltage-clamp studies of HEK cells transfected with Cav1.2Δ73 and β2- and α2δ1-subunits (plus pEGFP, Invitrogen) showed an absence of inward currents with 5 mM external Ca2+, whereas cells transfected with full-length-Cav1.2 plus β2- and α2δ1-subunits exhibited robust Ca2+ currents (Fig. 2, C and D).

Cav1 in A7r5 Smooth Muscle Cells

Previous studies demonstrated that A7r5 cells express DHP-sensitive Ca2+ currents (18), with Cav1.2 being the primary α1-subunit expressed (data not shown). Accordingly, we evaluated the suitability of A7r5 as an ASMC surrogate for addressing the functional effects of Cav1.2Δ73 subunits. Conventional RT-PCR and sequencing revealed Cav1.2Δ73 (20% of all Cav1.2 α1 transcripts) in RNA isolated from A7r5 cells (Fig. 3A). The pharmacology of Ca2+ currents in A7r5 cells was found to be similar to that in SMA myocytes (17). A major portion (80–90%) of the Ca2+ currents could be inhibited by 1 μM nifedipine, 10 μM verapamil, or 0.5 mM Cd2+ (Fig. 3B). Furthermore, complete inhibition of DHP-sensitive Ca2+ currents in A7r5 cells revealed a residual current of variable amplitude with the characteristics of a low-voltage-activated, T-type Ca2+ current (0.37 ± 0.05 pA/pF, n = 12; Fig. 3B), which SMA myocytes do not possess. For comparison, peak total current measured in low-passage A7r5 cells averaged 2.6 ± 0.4 pA/pF (n = 38). Currents measured with 2 mM extracellular Ca2+ from SMA myocytes and A7r5 cells (in the presence of amiloride) were similar in appearance (Fig. 3C) and had an identical voltage dependence when normalized by the peak value for each cell (Fig. 3D).

Cav1.2Δ73 Protein Is Made in A7r5 Cells

While Cav1.2Δ73 protein can be made in HEK-293 cells, can it be made in a smooth muscle cell context as well? A7r5 cells were transfected with the two Cav1.2Δ73 constructs, and 24–48 h later, decoration of Cav1.2Δ73-IREs-CD8-transfected cells with CD8 Dynabeads could be demonstrated (Fig. 4A), as could expression of V5-tagged protein in A7r5 cells transfected with Cav1.2Δ73-V5/ his by immunofluorescence (Fig. 4B). Western blots of lysates from A7r5 cells probed with an anti-Cav1.2 antibody showed a protein band of a slightly smaller size (left arrow) was also found in FuGENE HD-only-treated A7r5 cells and cells transfected with pIREs-CD8 alone (Fig. 4C). The Cav1.2Δ73 bands

Fig. 2. Expression of Cav1.2Δ73 in human embryonic kidney (HEK-293) cells. A: HEK-293 cells transfected with Cav1.2Δ73-IREs-CD8 and decorated with CD8-labeled beads (top) and immunofluorescence detection of V5 in cells transfected with Cav1.2Δ73-V5/ his (bottom). B: Western blot of lysates from cells treated with FuGENE HD only or transfected with pIREs-CD8, Cav1.2Δ73-IREs-CD8, and Cav1.2Δ73-V5/ his probed with Cav1.2 and V5 antibodies. C: representative whole cell current recorded from cells transfected with full-length Cav1.2 (•) or Cav1.2Δ73 (○), with β2- and α2δ1-subunits, plus enhanced green fluorescent protein (EGFP). Horizontal and vertical bars represent 20 ms and 100 pA, respectively. D: voltage dependence of peak current normalized by cell capacitance from cells transfected with full-length Cav1.2 (n = 8) or Cav1.2Δ73 (n = 12) plus accessory subunits. Values are means ± SE.
In transfected cells were larger than the presumed endogenous protein bands but were consistent with the size added by the constructs. These results suggest that small amounts of Cav1.2Δ73 protein are present in A7r5 cells that are enhanced by exogenous expression.

**Cav1.2Δ73 Expression Inhibits Endogenous Ca\(^{2+}\) Current in A7r5 Cells**

To address the functional effect of Cav1.2Δ73 expression on endogenous Ca\(^{2+}\) channel function, we transfected A7r5 cells with the Cav1.2Δ73-IRES-CD8 construct. On each coverslip, CD8 Dynabead-decorated and -undecorated cells were selected for voltage-clamp analysis of whole cell Ca\(^{2+}\) currents (Fig. 5A). In preliminary studies, we found that Ca\(^{2+}\) current density varied inversely with the number of CD8 beads decorating a cell, suggesting a graded inverse relation between Ca\(^{2+}\) current and Cav1.2Δ73 expression (not shown). Accordingly, for consistency, cells were selected with four to six attached beads. A comparison of current-voltage relations determined in the presence of amiloride to inhibit T-type currents (Fig. 5B) shows significantly smaller Ca\(^{2+}\) current in cells decorated with CD8 Dynabeads (0.8 ± 0.2 pA/pF, n = 21) than in undecorated (i.e., control) cells (2.7 ± 0.3 pA/pF, n = 26). Current-voltage data were used to calculate whole cell conductance, and a comparison showed no significant differences in the voltage dependence of normalized conductance between the two groups of cells (Fig. 5C). A7r5 cells were also transfected with the pIRES-CD8 construct without Cav1.2Δ73 (CD8 alone), and whole cell Ca\(^{2+}\) current density and conductance in CD8-positive cells (2.7 ± 0.4 pA/pF, n = 17) was not significantly different from Ca\(^{2+}\) current density and conductance in cells treated with empty vector, cells treated with FuGENE only, or untreated control cells. This indicates that overexpression of CD8 alone is not responsible for the inhibition of Ca\(^{2+}\) current.

**Effects of Cav1.2Δ73 on A7r5 Ca\(^{2+}\) Current Are Specific**

To address the question of the specificity of the effect of Cav1.2Δ73 on whole cell Ca\(^{2+}\) current (i.e., Cav1.2-containing channels), we expressed an equivalent, but unrelated, “truncated” Ca\(^{2+}\) channel subunit in A7r5 cells. RT-PCR analysis revealed the absence of Cav2.3 \(\alpha_1\) mRNA in A7r5, as well as SMA, cells (Fig. 6A). Therefore, we generated an analogous truncated form of Cav2.3 \(\alpha_1\) (aa 1–654) from rat brain RNA and cloned it into the MCS A of pIRES-CD8. Whole cell Ca\(^{2+}\) currents (Fig. 6B) measured in A7r5 cells transfected with Cav2.3Δ73-IRES-CD8 and decorated with CD8 Dynabeads (2.4 ± 0.2 pA/pF, n = 14) were not significantly different from Ca\(^{2+}\) currents measured in control (untreated, undecorated, or empty vector-treated) A7r5 cells (Fig. 6C). These results suggest that the effects of Cav1.2Δ73 on A7r5 Ca\(^{2+}\) current are specific.

In untransfected cells studied without amiloride, low-voltage-activated, T-type Ca\(^{2+}\) current could be identified as a low-voltage current “bump” in the voltage ramp response (Fig. 7), but current levels were small and varied considerably from cell to cell. In some cells transfected with Cav1.2Δ73-V5/his, we observed the complete inhibition of L-type currents, while smaller-amplitude T-type currents remained (Fig. 7). T-type currents in the absence of L-type currents were not observed in
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control or untreated cells (>200). In cells transfected with Cav1.2Δ73-V5/his, average whole cell current density was significantly smaller at all voltages greater than but not less than −20 mV (Fig. 7). Conventional RT-PCR showed the expression of Cav3.1 and Cav3.3 in RNA from A7r5 cells that may represent the molecular basis for the T-type currents in these cells (data not shown).

Cav1.2Δ73 Does Not Span the Plasma Membrane

Cell surface proteins of HEK cells transfected with Cav1.2Δ73-V5/his and β₂ and α₃δ₁-subunits were biotinylated and isolated with streptavidin-coated agarose beads and, along with nonbiotinylated proteins and whole cell lysates, used for Western blot analysis of Cav1.2Δ73 expression. Strong anti-V5 signals were found in the whole cell and nonbiotinylated (intracellular) fraction, but not in the biotinylated (transmembrane) fraction (Fig. 8). Reprobing these blots with an anti-β-actin antibody (catalog no. A2066, Sigma Chemical) showed the presence of actin in the whole cell lysate and nonbiotinylated fraction, but not in the biotinylated fraction (Fig. 9), confirming that the reagent did not enter the cells and label intracellular proteins.

Cav1.2Δ73 Localizes to the ER

To determine the cellular localization of Cav1.2Δ73, we transfected HEK cells with Cav1.2Δ73-V5/his plus β₂- and α₃δ₁-subunits. Cells selected with neomycin were propagated to 90% confluence, passaged, and transfected with a green fluorescent protein (GFP)-tagged ER marker in baculovirus particles (ER Organelle Lights). Immunofluorescence showed that 100% of cells expressing V5 also expressed GFP (orange, Fig. 9A). Some cells expressed GFP alone, with no evidence of Cav1.2Δ73-V5/his expression, which was expected on the basis of the transfection efficiency of FuGENE HD. Images from Cav1.2Δ73-V5/his-expressing cells not transfected with ER Organelle Lights showed no apparent “colocalization”

Fig. 6. Effects of Cav2.3Δ73 expression on Ca²⁺ currents in A7r5 cells. A: ethidium bromide-stained agarose gel shows expression of Cav2.3 in rat brain (rB), but not in RNA from SMA myocytes or A7r5 cells. Rat genomic DNA (rG) was used as a PCR control. B: whole cell Ca²⁺ currents recorded in the presence of amiloride from a representative cell transfected with Cav2.3Δ73-ires-CD8 and decorated with CD8-labeled beads (inset). Horizontal and vertical bars represent 20 ms and 100 pA, respectively. C: whole cell current density in cells transfected with Cav2.3Δ73-ires-CD8 and FuGENE HD-only controls. Values are means ± SE.

Fig. 5. Effects of Cav1.2Δ73 expression on Ca²⁺ currents in A7r5 cells. A: representative whole cell currents recorded in the presence of amiloride from cells transfected with Cav1.2Δ73-ires-CD8 but not decorated with CD8 beads (nonexpressing control), cells transfected with Cav1.2Δ73-ires-CD8 and decorated with CD8 beads (expressing), and cells transfected with pRES-CD8 (CD8 alone). Horizontal and vertical bars represent 20 ms and 100 pA, respectively. B: voltage dependence of peak current normalized to cell capacitance for the 3 groups. C: voltage dependence of normalized whole cell Ca²⁺ conductance calculated from peak current (gₚk) in each cell as follows: \( g = \frac{g_{\text{max}} V}{V - E_{\text{rev}}} \), where \( g_{\text{max}} \) is maximum conductance and \( E_{\text{rev}} \) is reversal potential. Values are means ± SE.
A7r5 cells transfected with Cav1.2Δ73-IRES-CD8 and decorated with CD8 Dynabeads exhibited a large reduction in peak Ca\(^{2+}\) currents compared with undecorated cells on the same coverslip, cells transfected with pIRES-CD8 only, and plasmid-free FuGENE HD-treated cells. It is not likely that the smaller currents in the CD8 bead-decorated cells are the result of a nonspecific effect of saturation of endogenous protein expression by an exogenous construct, as peak Ca\(^{2+}\) current density in CD8 bead-decorated A7r5 cells transfected with pIRES-CD8 without Cav1.2Δ73 or with Cav2.3Δ73-IRES-CD8 was not significantly different from that in undecorated or untreated cells. Furthermore, T-type Ca\(^{2+}\) currents were found in some A7r5 cells transfected with Cav1.2Δ73-V5/His in the complete absence of L-type current, which was not observed in any untreated or control cells. This provides further evidence against a nonspecific effect, as it would be unlikely for one channel type to be suppressed (L-type), but not the other (T-type).

**Mechanism of Cav1.2Δ73 Effect**

From the basic definition of whole cell current (I = i\(*N\)*Po), the inhibitory effect of Cav1.2Δ73 could be the result of reduced single-channel current (i), a decreased number of functional channels (N), or an altered voltage dependence of channel open probability (Po). The voltage dependence of whole cell conductance in A7r5 cells expressing Cav1.2Δ73-IRES-CD8 (with

![Fig. 7. Ca\(^{2+}\) currents in A7r5 cells transfected with Cav1.2Δ73-V5/His. A: current response to a voltage ramp from -80 to +40 mV shows a “bump” at low voltage (arrow) due to T-type channels. B: currents recorded from a cell 24 h after transfection with Cav1.2Δ73-V5/His (○) show only rapidly inactivating, low-voltage-activated Ca\(^{2+}\) current compared with a control (untransfected) cell, which is a mixture of the 2 types (●). Horizontal and vertical bars represent 20 ms and 50 pA, respectively. C: summary of average peak current density-voltage data from control cells (pEGFP only) and cells 24 h after transfection with Cav1.2Δ73-V5/His plus pEGFP (Δ73-V5). Values are means ± SE.](image)

**DISCUSSION**

The results of these experiments demonstrate for the first time that a naturally occurring truncated Cav1.2 α\(1\)-subunit isoform (Cav1.2Δ73) produced by alternative splicing can suppress endogenous DHP-sensitive Ca\(^{2+}\) current (Cav1.2) in a smooth muscle (A7r5) cell context. The presence of this truncated transcript has been demonstrated in human fibroblasts, brain, and heart (33, 35), as well as in rat cerebral artery myocytes (4). It appears to arise from the use of an alternative splice acceptor site removing the proximal 73 nt of exon 15 (35). The observations that this transcript is widely distributed in cardiovascular tissue, as well as in brain, of rat and human, that its primary sequence is 100% conserved among all mammalian species [Basic Local Alignment Search Tool (BLAST) against the Nucleotide Collection of the National Center for Biotechnology Information Databases], and that it arises by alternative splicing suggest that the abundance of this transcript may be regulated (3).

**A7r5 Cell Model**

A variety of heterologous expression systems have been used to assess the function of Ca\(^{2+}\) channels, including Xenopus oocytes, mammalian and insect cells, native myocytes in short-term culture, and intact vascular segments. We chose to utilize a cell line derived from neonatal rat aorta for several reasons. A7r5 cells are of arterial origin, they stably express functional L-type Ca\(^{2+}\) channels, they have been employed in previous studies, and they are well characterized (18). Molecu

![Fig. 8. Cav1.2Δ73-V5/His is not inserted into the plasma membrane. Western blots of cell surface (Beads) and intracellular protein (Supe) fractions of HEK cells transfected with Cav1.2Δ73-V5/His plus β2 and α\(2/β\) accessory subunits, along with whole cell lysates (Lysate). Cells were treated with (Biotinylated) or without (Control) biotin cross-linking reagent. Expression of V5-tagged Cav1.2Δ73 and actin was found in the whole cell lysate and nonbiotinylated fractions, but not in the biotinylated fraction.](image)
reduced current amplitude) was not different from that of control cells. This suggests that the voltage dependence of $P_o$ is unchanged in these cells, that the presence of Cav1.2Δ73 does not alter Ca$^{2+}$ current gating, and that functional channels likely have the same molecular composition as controls. Truncated subunits might be trafficked to the plasma membrane, where they could interact with full-length subunits (channels) and decrease single-channel current or the number of functional channels. However, biotinylation and immunofluorescence studies suggest that the truncated subunits are not inserted into or located at the plasma membrane but are localized to the ER, where they are unlikely to have a direct effect on whole cell currents. If the molecular composition of the channels is unchanged by expression of Cav1.2Δ73-IRE-CD8 and the truncated subunit is not present at the plasma membrane, it is unlikely that single-channel conductance would be altered. This leaves a reduction in the number of functional, plasma membrane-localized channels as the likely explanation.

A number of truncated Cav $\alpha_1$-subunits have been identified that arise secondary to mutations (13, 19) or changes in translation initiation (27, 41) and lead to the production of nonfunctional truncated $\alpha_1$-subunits. Many of these act as dominant-negative regulators of full-length Cav function (19, 28). The effects of truncated Cav2.1 and Cav2.2 $\alpha_1$-subunits have been studied in the greatest detail. These studies show that truncated and full-length subunits are synthesized but strongly associate in the ER, where they are retained. As a result, some full-length $\alpha_1$-subunits are unable to fold properly, to associate with accessory subunits, or to be trafficked from the synthetic pathway to the plasma membrane (13, 19, 28). Our finding of colocalization of Cav1.2Δ73 subunits with an ER marker and the conclusion that the number of functional channels is reduced support the hypothesis that Cav1.2Δ73 subunits act in a manner similar to that of truncated Cav2 $\alpha_1$-subunits.

Page et al. (28) demonstrated that functional expression of Cav2.1 and Cav2.2 was strongly inhibited by coexpression of truncated constructs containing domain I. Subsequent studies indicated that an 11-amino acid sequence (plus Arg52 and Arg54) within the NH2 terminus was critical for the dominant-negative inhibitory effects of these truncated constructs (29). Interestingly, Bannister et al. (1) recently demonstrated that exogenous expression of NH2-terminal exon 1b or 1c fragments in cerebral artery-derived myocytes in short-term culture also decreased expression of endogenous Cav1.2 channels. It is tempting to conclude from these studies that truncated fragments of $\alpha_1$-subunits containing the NH2 terminus generally act to inhibit functional activity of their full-length counterparts by a direct interaction and resultant intracellular retention followed by degradation. However, Ebihara et al. (8) showed that a truncated Cav1.2 construct lacking domain I and a part of domain II inhibited full-length Cav1.2 currents when coexpressed in Xenopus oocytes by a dominant-negative mechanism. Together, these results suggest that multiple mechanisms may exist by which truncated $\alpha_1$-subunits can inhibit functional activity of their full-length counterparts.

**Physiological Significance**

The complete open reading frame of the Cav1.2 $\alpha_1$-subunit has 90% identity between rat, human, and rabbit and 95% identity between rat and mouse. BLAST of the 73-nt sequence against the Nucleotide Collection of National Center for Biotechnology Information databases shows that this sequence is 100% identical across all mammalian species from mouse, rat, rabbit, and guinea pig to cow, horse, and human. By comparison, the alternatively spliced exon 9*, which is highly expressed in ASMCs (4, 10, 25), is only 94% identical between rat and mouse, two closely related species. The high level of conservation of the 73-nt sequence across species suggests that the sequence has fundamental, intrinsic importance.

Truncated Cav $\alpha_1$-subunit proteins have been shown to arise naturally as a result of alternative splicing (41) or through the use of alternative transcriptional start sites (27). In some cases,
these truncated isoforms have been shown to be developmentally regulated (41) and to play an important role in normal development by suppressing the activity of full-length isoforms (27). Our results suggest that Cav1.2Δ73 is a naturally occurring truncated transcript produced by alternative splicing that is widely distributed and compatible with “normal” cell function. As alternative splicing can occur co- or posttranscriptionally (40) and transcription is highly regulated (39), it is reasonable to suggest that the splicing that produces Cav1.2Δ73 is also regulated. This raises the possibility that Cav1.2Δ73 may function as a regulator of Cav1.2 α1-subunit plasma membrane expression and play a role in fine-tuning its functional activity in cooperation with other regulatory mechanisms (30). Notably, with heterozygous knockout of Cav1.2 in the adult mouse heart, Cav1.2 currents in isolated cardiomyocytes are unaffected, despite a 42% decrease in mRNA levels, suggesting that (in this case) functional activity can be effectively regulated by posttranscriptional mechanisms (31).

The suggestion that a nonfunctional gene product produced naturally by alternative splicing may play a role in the regulation of full-length protein activity is not new (15). There are numerous examples in the literature where such a role has been demonstrated for truncated isoforms of G protein-coupled receptors (37), nuclear receptors (36), and other ion channels (26), as well as other proteins (16). In many of these cases, critical domains are lost as a result of the alternative splicing, rendering such truncated isoforms nonfunctional, leading to their retention within the cell, where they form heterodimers with full-length isoforms. This reduces trafficking of full-length functional proteins to the plasma membrane (or to other cell compartments), thereby decreasing the protein’s functional activity (26, 36). In some cases, the abundance of the spliced isoform is directly regulated, contributing to the regulation of full-length isoform activity (12, 16). This literature provides support for the suggestion of a possible regulatory function of Cav1.2Δ73 subunits.

Limitations

Several limitations of this study should be acknowledged. First, A7r5 cells are not native SMCs but, rather, an immortalized cell line derived from embryonic rat aorta (14). As designed, the studies reported here require ≥3 days, so removal of arteries and/or cells from their native microenvironment (e.g., their neurohumoral and mechanical input) for this purpose is likely to alter expression of Cav1 subunits (24), such that they may not be in a steady state during the time required to perform these experiments. This could complicate interpretation of the results. A7r5 cells stably express the Cav1.2 α1-subunit, as well as accessory subunits, thereby eliminating stability of expression as an issue. Furthermore, the primary goal of this work was to obtain “proof-of-principle” for an inhibitory action of Cav1.2Δ73 on the functional activity of Cav1.2 channels, which has been demonstrated. Clearly, future experiments are required to confirm a similar role of Cav1.2Δ73 in native myocytes, as well as to determine factors or conditions that modify the abundance of Cav1.2Δ73 subunits.

Expression studies were performed using a cell population, not single smooth muscle cells. It is not clear if Cav1.2Δ73 is expressed in every cell in a resistance artery segment or in every vascular bed. However, its presence in other tissues, including cerebral resistance arteries, has been demonstrated (4, 33, 35), but whether it is expressed in every cell or in a distinct subset of cells remains unclear. While the expression of specific transcripts of Cav1.2 subunits, including splice variants, may vary among cells within the media of an artery segment, it is the integrated activity of the total population of cells that determines the functional properties of a resistance artery.

Finally, biochemical studies using A7r5 cells to explore the mechanism of action of Cav1.2Δ73 in detail were not possible because of the low transfection efficiency (5–10%) of FuGene HD in these cells. As a result, the contribution from untransfected cells would have swamped that of positively transfected cells, preventing meaningful analysis. We did attempt the use of a number of different transfection methods, including nanoparticle delivery and reverse permeabilization, but we had no better success.

GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

R.H.C. and S.J.F. performed the experiments; R.H.C. and S.J.F. analyzed the data; R.H.C. and S.J.F. interpreted the results of the experiments; R.H.C. drafted the manuscript; R.H.C. and S.J.F. edited and revised the manuscript; R.H.C. and S.J.F. approved the final version of the manuscript.

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