Splice Variant Specific Modulation of CaV1.2 Calcium Channel by Galectin-1 Regulates Arterial Constriction

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Rationale: CaV1.2 channels are essential for excitation–contraction coupling in the cardiovascular system, and alternative splicing optimizes its role. Galectin-1 (Gal-1) has been reported to regulate vascular smooth muscle cell (VSMC) function and play a role in pulmonary hypertension. We have identified Gal-1 multiple times in yeast 2-hybrid assays using the CaV1.2 I–II loop as bait.

Objective: Our hypothesis is that Gal-1 interacts directly with CaV1.2 channel at the I–II loop to affect arterial constriction.

Methods and Results: Unexpectedly, Gal-1 was found to selectively bind to the I–II loop only in the absence of alternatively spliced exon 9*. We found that the current densities of CaV1.2α9* channels were significantly inhibited as a result of decreased functional surface expression due to the binding of Gal-1 at the export signal located on the C-terminus of exon 9. Moreover, the suppression of Gal-1 expression by siRNA in rat A7r5 and isolated VSMCs produced the opposite effect of increased ICa,L. The physiological significance of Gal-1 mediated splice variant-specific inhibition of CaV1.2 channels was demonstrated in organ bath culture where rat MAs were reversibly permeabilized with Gal-1 siRNA and the arterial wall exhibited increased K+–induced constriction.

Conclusion: The above data indicated that Gal-1 regulates ICa,L via decreasing the functional surface expression of CaV1.2 channels in a splice variant selective manner and such a mechanism may play a role in modulating vascular constriction. (Circ Res. 2011;109:1250-1258.)

Key Words: CaV1.2, galectin-1, vascular smooth muscle cell, arterial constriction

The CaV1.2 L-type calcium channels (LTCCs) are the primary conduits for Ca2+ influx that lead to initiation of numerous physiological processes, including muscle excitation–contraction coupling, neurotransmitter release, gene expression, and hormone secretion. Under optimal conditions, Ca2+ influx through the activated CaV1.2 channels in the vascular smooth muscle cells (VSMCs) tightly influences the contractility of small arteries and arterioles, and maintains the myogenic tone of resistance vessels. However, if this process goes awry during the pathogenesis of hypertension, an increase in Ca2+ influx may contribute to the development of an abnormal vascular tone and result in elevated peripheral vascular resistance. Of the 4 subunits (α1, β, α2δ, and γ) that composed the calcium channels, the CaVα1 subunit not only forms the aqueous pore but also possesses the voltage sensor, gating apparatus and sites for channel regulation by second messengers, drugs, and toxins. Within the intracellular linker region between homologous domains I and II of α1 subunit (I–II loop) is the α1–subunit interaction domain (AID), a site where the auxiliary CaVβ subunit binds to traffic α1 subunit to cell membrane. Moreover, alternative splicing at the CaV1.2 I–II loop produces a subpopulation of CaV1.2 channel splice variant that contain exon 9*, which is important for vasotone maintenance in blood vessels (Figure 1A). To date, we and others have identified and characterized 3 predominant CaV1.2 splice combinations that are found in arterial smooth muscles, namely, CaV1.2γ7WT (1-8-9*-33), CaV1.2SM (1-8-9*-Δ33), and CaV1.2β (1-8-9*-33).

Galectins are a family of carbohydrate-binding proteins with an affinity for β-galactosides. Galectin-1 (Gal-1) plays a number of roles in physiological and pathological situations. While extracellular Gal-1 regulates cell–cell and cell–matrix interactions, the immune response, apoptosis, and neoplastic transformation, intracellular Gal-1 regulates the cell cycle, RNA splicing, and transcription. It has been reported that Gal-1 is a modulator of vascular functions, including VSMC differentiation, proliferation, and migra-
tion. Furthermore, Gal-1 has been implicated in the regulation of blood pressure, which showed that Gal-1−/− null mice developed higher pulmonary blood pressure under acute hypoxia compared with wildtype mice.18

In this study, we unexpectedly discovered that Gal-1 binds to the subpopulation of CaV1.2 channels that are devoid of alternatively spliced exon 9*. The interaction of Gal-1 with CaV1.2 channels clearly inhibited channel function and in resistance arteries resulted in lower constriction of the smooth muscles.

Methods

Yeast 2-Hybrid Assay

Yeast 2-hybrid screen was performed using the cytoplasmic I–II loop of human CaV1.2 (in a pGBK7 vector) as bait in an X α-Gal in-gel assay. A total of 75 quadruple DO and 25 triple DO plates were used for the assay. A human aorta cDNA library (Clontech #HL4040AH) was used for screening. Colonies were observed after transformation from day 5 to day 8.

GST Pull-Down Assay

GST pull-down assays were performed using glutathione sepharose 4B GST beads (GE Healthcare, Little Chalfont, UK) to isolate purified GST linked to different exons of CaV1.2 I–II loop or truncated exons. The immobilized GST or GST-fusion proteins were incubated with cell lysates prepared from A7r5 cells. Glutathione-bound proteins were eluted by boiling at 95°C in 2×SDS-sample buffer for 5 minutes, and detected by immunoblotting with anti-Gal-1 (Invitrogen; 1:2000 dilutions) or anti-GST (Santa Cruz; 1:5000 dilutions) antibodies.

Statistical Methods

Averaged data were reported as mean±SEM. Statistical significance was analyzed using a Student unpaired t test or 1-way analysis of variance.

**Non-standard Abbreviations and Acronyms**

AID: α1-subunit interaction domain
CaMKII: calcium/calmodulin-dependent kinase II
DHP: dihydropyridine
ER: endoplasmic reticulum
ERAD: endoplasmic reticulum-associated protein degradation
Gal-1: Galectin-1
HEK: human embryonic kidney
LTCC: L-type voltage-dependent calcium channel
MA: mesenteric artery
NT: nontargeting
PSS: physiological saline solution
QON: ON-gating currents
RP: reversible permeabilization
siRNA: small interfering RNA
STIM1: stromal interacting molecule 1
VSMC: vascular smooth muscle cell

**Figure 1. Gal-1 binds to I–II loop of CaV1.2 α1c subunit.** A, Schematic diagram of the CaV1.2 calcium channel shows the I–II loop and alternatively spliced exon 9*. Amino acid sequences of human CaV1.2 α1c I–II loop exon 9, 9*, and 10 are indicated in the lower panel with AID domain highlighted in blue and di-acidic motifs of ER export signal in red. B, Western blot analysis of GST pull-down assays demonstrating pull down of Gal-1 only when the GST-fusion protein contains exon 9 in the absence of exon 9*. C, Diagrammatic representation of the full-length and the N- or C-terminal truncated constructs of exon 9 (upper panel); GST pull-down assay showed that Gal-1 was isolated only when the C-terminus of exon 9 is present (lower panel). D, Diagrammatic representation of the full-length and the 5 3-aa deletional constructs of exon 9 (left panel); GST pull-down assay showed the requirement of the ER export signal (red column) for pulling down Gal-1 (right panel).
variance, followed by Bonferroni’s method for post hoc pairwise multiple comparisons. Statistical significance was set at \( P < 0.05 \).

An expanded Methods section is available online at http://circres.ahajournals.org.

**Results**

**Gal-1 Binds Preferentially to CaV1.2 I–II Loop Without Exon 9**

To identify proteins that interact with the I–II loop of CaV1.2 channels, we performed yeast 2-hybrid assay and after screening \( 3.5 \times 10^6 \) independent clones from human aorta cDNA library, we found 7 positive clones that were confirmed to be human Gal-1. The bait contained the CaV1.2 I–II loop that includes exon 9 and 10, but without the alternative spliced exon 9*. To clarify the interacting domain of Gal-1 with I–II loop of CaV1.2 channel, we cloned exon 9, exon 9*, exon 10, exons 9+10, and exons 9+9*+10 into pGEX4T-1 expression vector. Using these GST fusion proteins as baits in GST pull-down assays, we found that Gal-1 bound strongly to exon 9 and exons 9+10 GST fusion proteins. The interaction between Gal-1 and exon 9+9*+10 was weak, as only a faint band of Gal-1 was detected (Figure 1B).

To further investigate the binding site on exon 9, we generated 2 truncated AID-containing constructs to perform GST pull-down assays. We found that Gal-1 can bind to the portion of exon 9 C-terminus to the AID, but not to the N-terminus (Figure 1C). To delineate the binding region, we performed deletional experiments in which 3 amino acids were deleted sequentially from the C-terminus of exon 9. Five deletional exon 9 constructs were generated, and we found that amino acids at positions 40 to 54 of exon 9 are important as binding site for Gal-1 (Figure 1D).

**Gal-1 Colocalizes With CaV1.2 in Arterial Smooth Muscle**

The inclusion or exclusion of splice variant exon 9* could be found in human and rat arterial tissues (Figure 2A). Similarly, although CaV1.2 channels are widely expressed in the cardiovascular system, reverse-transcription polymerase chain reaction

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**Figure 2. Coexpression of Gal-1 and CaV1.2 in arterial smooth muscle.**

**A,** Schematic diagram showing the locations of the PCR primers designed to amplify and detect rat or human CaV1.2 I–II loop inclusive of or in the absence of exon 9* in arterial tissues. PCR products were separated in 2% agarose gel, and both rat and human arteries contained an almost equal amount of products with the inclusion (upper band) or exclusion (lower band) of exon 9*. **B,** RT-PCR products of Gal-1 mRNA were detected in the human arteries but not in the human heart. Similarly, Gal-1 protein as detected by Western blot analysis was observed in the rat arteries but not the heart tissues. **C,** Immunofluorescence staining to determine colocalization of \( \alpha \)-SM actin, CaV1.2 channels, and Gal-1 in smooth muscles was carried out using anti-\( \alpha \)-SM actin (left top, 1:100 dilutions), anti-CaV1.2 \( \alpha_{1C} \) antibody (left bottom, 1:100 dilutions), and anti-Gal-1 antibody (middle, 1:100 dilutions) in rat mesenteric artery. The right panel shows the merged image.
Gal-1 Selectively Regulates the Function of CaV1.2 Channel Splice Variants

To date, there are 3 reported dominant splice combinations among the 3 CaV1.2 splice variants to explain the selective inhibition by Gal-1: lack of exon 9* in the CaV1.277WT channel, and CaV1.2 isoforms: CaV1.2SM and CaV1.2b. There were no significant differences in I_{Ca,L} when HEK 293 cells were cotransfected using CaV1.2SM (Figure 3C) or CaV1.2b (Figure 3D) channels with Gal-1. The steady state activation and inactivation potentials and the rate of recovery from inactivation were also not obviously changed (Online Figure IIA and IIC and Online Tables II and III). However, the major difference observed among the 3 CaV1.2 splice variants to explain the selective inhibition by Gal-1 is the lack of exon 9* in the CaV1.277WT channels. To further validate this observation, we examined the effect of Gal-1 on another CaV1.2 splice variant that does not contain exon 9*: CaV1.2CM. This is a predominant cardiac isoform, and we found that Gal-1 significantly decreased the current density (Figure 3E). For CaV1.2CM, Gal-1 also right-shifted the steady state inactivation while not affecting the steady

The inhibition by Gal-1 was also observed when Ba^{2+} was used as the charge carrier (Figure 3B), which indicated that the inhibition by Gal-1 was calcium independent. However, channel electrophysiological properties such as the steady state activation and inactivation potentials, and the rate of recovery from inactivation remained similar (Online Figure IIA and Online Tables II and III).

To further determine the functional interactions between Gal-1 and CaV1.2 channels, we tested the other 2 smooth muscle isoforms: CaV1.2SM and CaV1.2b. There were no significant differences in I_{Ca,L} when HEK 293 cells were cotransfected using CaV1.2SM (Figure 3C) or CaV1.2b (Figure 3D) channels with Gal-1. The steady state activation and inactivation potentials and the rate of recovery from inactivation were also not obviously changed (Online Figure IIB and IIC and Online Tables II and III). However, the major difference observed among the 3 CaV1.2 splice variants to explain the selective inhibition by Gal-1 is the lack of exon 9* in the CaV1.277WT channels. To further validate this observation, we examined the effect of Gal-1 on another CaV1.2 splice variant that does not contain exon 9*: CaV1.2CM. This is a predominant cardiac isoform, and we found that Gal-1 significantly decreased the current density (Figure 3E). For CaV1.2CM, Gal-1 also right-shifted the steady state inactivation while not affecting the steady

Gal-1 Selectively Regulates the Function of CaV1.2 Channel Splice Variants

To date, there are 3 reported dominant splice combinations found in blood vessels, and these variants differ in the utilization of alternative splice sites at the N-terminus, I–II loop, and domain IV S3–S4 regions. There were no significant differences in I_{Ca,L} when HEK 293 cells were cotransfected using CaV1.2SM (Figure 3C) or CaV1.2b (Figure 3D) channels with Gal-1. The steady state activation and inactivation potentials and the rate of recovery from inactivation were also not obviously changed (Online Figure IIB and IIC and Online Tables II and III). However, the major difference observed among the 3 CaV1.2 splice variants to explain the selective inhibition by Gal-1 is the lack of exon 9* in the CaV1.277WT channels. To further validate this observation, we examined the effect of Gal-1 on another CaV1.2 splice variant that does not contain exon 9*: CaV1.2CM. This is a predominant cardiac isoform, and we found that Gal-1 significantly decreased the current density (Figure 3E). For CaV1.2CM, Gal-1 also right-shifted the steady state inactivation while not affecting the steady

(RT-PCR) and Western blot analysis showed that Gal-1 was expressed only in the vascular smooth muscle tissue, but not in the heart tissue (Figure 2B). We have also investigated the presence of Gal-1 with CaV1.2 channels in rat MA by immunofluorescence staining and found strong colocalization of Gal-1 and CaV1.2 channels in the arterial wall. Moreover, Gal-1 and α-SM actin were colocalized in rat MA, indicating that Gal-1 is selectively expressed in the smooth muscle layer of arteries (Figure 2C).
state activation and rate of recovery from inactivation (Online Figure IID and Online Tables II and III).

Gal-1 Decreased the Functional Surface Expression of CaV1.2\textsuperscript{77WT} Channels

To determine the mechanism by which Gal-1 decreases CaV1.2\textsuperscript{77WT} current density, we characterized the ON-gating currents (\(Q_{\text{ON}}\)) reflecting the capacitative voltage-sensor movements on depolarization during channel gating by depolarizing cells to positive potentials at which no ionic inward and outward currents were observed when holding at \(V_{\text{rev}}\). It is found that coexpression of Gal-1 could reduce \(Q_{\text{ON}}\) by \(\approx45\%\) in CaV1.2\textsuperscript{77WT} channels, suggesting a lower-level functional expression of the channels on the membrane (Figure 4B). Furthermore, the expression of surface CaV1.2\textsuperscript{77WT} channel protein was decreased about 50\% when cotransfected with Gal-1 (Online Figure III). However, there was no significant difference in \(Q_{\text{ON}}\) in the other 2 smooth muscle CaV1.2 channels that contained exon 9\* (Figure 4C and 4D). Significantly, CaV1.2\textsuperscript{CM} channels that lack exon 9\* have also decreased \(Q_{\text{ON}}\) by \(\approx45\%\) when coexpressed with Gal-1 (Figure 4E). Interestingly, when we cotransfected different amounts of Gal-1 with constant amounts of CaV1.2\textsuperscript{77WT} subunits, we showed that Gal-1 dose-dependently inhibits CaV1.2 calcium currents (Online Figure IV). Taken together, these findings strongly suggest that binding of Gal-1 to the I–II loop of CaV1.2 channels occurred in the absence of exon 9\*. The inhibition of CaV1.2\textsuperscript{77WT} current density is possibly mediated by downregulation of surface expression of the channels via competition between Gal-1 and \(\beta2\alpha\) for binding to the I–II loop of CaV1.2 or the masking of the endoplasmic reticulum (ER) export signal by Gal-1.

siRNA Mediated Knock-Down of Gal-1 Increases Calcium Channel Currents of VSMCs

To strengthen the hypothesis that Gal-1 reduces CaV1.2 currents, we performed the reverse experiment and decreased...
Gal-1 expression by transfecting siRNA into rat aortic smooth muscle cell (SMC) line A7r5 or isolated rat aortic SMCs. After transfection of Gal-1 siRNAs into A7r5 cells and primary cultured VSMC cells, endogenous Gal-1 protein level was substantially decreased, while nontargeting (NT) siRNA had no effect on Gal-1 expression (Figure 5A and Online Figure VIA). Notably, I_{Ba,L} was increased by about 75% in A7r5 cells (Figure 5B) and 35% in isolated VSMCs (Figure 5C) after Gal-1 siRNA treatment compared with NT siRNA, respectively. However, the steady state inactivation (Online Figure VIB) and rate of recovery from inactivation (Online Figure VIC) in A7r5 cells were not altered by Gal-1 siRNA treatment (Online Table IV), which is consistent with the data obtained from experiments done in HEK 293 cells. These results indicate that knock-down of Gal-1 in VSMCs could up-regulate calcium currents.

As it has been reported that arterial SMCs also expressed CaV1.3 channels,20 we therefore used 2 concentrations of nifedipine to apply on A7r5 cells to assess the levels of expression of CaV1.2 and CaV1.3 currents on the basis of their different sensitivities to dihydropyridine (DHP).21 Here, we found that nifedipine inhibited the calcium currents similarly in 1 μmol/L or 10 μmol/L of nifedipine (Online Figure VIIA). Moreover, the increase in I_{Ba} after knock-down of Gal-1 expression by siRNA could be completely blocked by 1 μmol/L nifedipine (Online Figure VIB). These data indicate that the major calcium currents of VSMCs and the increase in I_{Ba} after Gal-1 knock-down are due to Ca^{2+} flux through the CaV1.2 and not the CaV1.3 channels.

Gal-1 Regulates Arterial Constriction

Calcium influx through CaV1.2 channels is important for vascular constriction; therefore, we next examined whether Gal-1 could affect vascular constriction. Reversible permeabilization (RP) procedure was used to knock down the expression of Gal-1 in rat MAs in organ bath culture. After 3 days of RP with Gal-1 siRNA, the Gal-1 expression was significantly decreased in rat mesenteric arterial (Figure 6A). Vascular constrictions were measured at different days after RP using the Living System.10,22 At day 3 after RP, the arteries subjected to Gal-1 siRNA treatment showed larger arterial wall constrictions in stepwise increases of external potassium ions ([K^+]_o) as compared to NT siRNA (Figure 6B and 6C), but there is no difference in the [K^+]_o EC_{50} between the Gal-1 siRNA treated and NT siRNA treated arteries (Online Table V). However, at days 1 and 2, Gal-1 siRNA had no significant effect on mesentery arterial wall constriction (Online Figure VIII). In summary, the absent of Gal-1 up-regulates CaV1.2 channel activity, resulting in vasoconstriction of the MAs.

Discussion

The CaV1.2 channels are the primary conduits for Ca^{2+} influx that trigger excitation–contraction coupling in small resistance arteries. Therefore, it is a logical target for homeostatic regulation of Ca^{2+} influx into a cell via multiple mechanisms, including posttranscriptional and posttranslational modifications or modulations by drugs or cytoplasmic interacting proteins.23,24 In this study, we found that (1) Gal-1 could bind to CaV1.2 I–II
loop; (2) Gal-1 decreased $I_{Ca,L}$ of Ca$_{1.2,39^+}$ by reducing the surface expression of Ca$_{1.2}$; and (3) knock-down of Gal-1 increased the $I_{Ca,L}$ in VSMCs and K$^+$-induced arterial constriction.

The Ca$_{1.2}$ channels, like other membrane channels and receptors, is a hetero-oligomeric complex that is also associated with signaling molecules, enzymes, or anchoring proteins. To date, several small proteins such as Ca$_{1.2}$ subunit, calmodulin, CaMKII (calcium/calmodulin-dependent kinase II), and STIM1 (stromal interacting molecule 1) have been found to bind to the α$_{1C}$ I–II loop or C-terminus to regulate Ca$_{1.2}$ channel functions. Adding to this list is Gal-1, and interestingly Gal-1 has also been shown to interact with other cell membrane channels or receptors to modulate their functions. Binding of Gal-1 to the renal epithelial Ca$^{2+}$ channels TRPV5 results in their accumulation on the plasma membrane, while the binding of Gal-1 to CD43 or CD45 on dendritic cells triggers cell activation and migration. Our data showed that Gal-1 inhibits Ca$_{1.2}$ currents via reducing the functional surface expression of the channels without affecting their electrophysiological properties. A possible mechanism is the competition of Gal-1 with Ca$_{1.2}$ subunit for binding to exon 9, the segment of the I–II loop where the AID resides. In the absence of Ca$_{1.2}$ subunit binding to the AID, the Ca$_{1.2}$ channels could be subject to proteasomal degradation via the endoplasmic reticulum-associated protein degradation (ERAD) complex. Di-acidic motifs (DxD, DxE, ExD, and ExE, where x is any amino acid) have been found to act as ER export signals in many membrane proteins. Recently, an α$_{1C}$ export signal was reported to be located at the C-terminus of exon 9 (DIDPENEDGDEEE), which is rich in di-acidic motifs, next to the AID domain. The report also showed that the Ca$_{1.2}$ subunit may interact with this ER export signal to affect the trafficking of the α$_{1C}$ subunits from the ER to surface membrane, as mutations generated in this region decreased functional Ca$_{1.2}$ surface expression and hence decreased the maximal gating currents. Here, we have demonstrated that the Gal-1 also binds to this ER export signal, which therefore provides another mechanistic understanding of how Gal-1 may mask the ER export signal to affect Ca$_{1.2,39^+}$ channel surface expression and current density.

Gal-1 was found to be involved in the attachment, spreading, and proliferation of VSMC. Notably, Gal-1 could inhibit VSMC spreading and migration via interactions with extracellular matrix proteins and α1β1 integrin; moreover, it has also been shown that Gal-1 inhibits VSMC adhesion by activating β1 integrin and phosphorylating focal adhesion kinase. Importantly, Ca$^{2+}$ influx through LTCC also mediates VSMC attachment, spreading, and migration. As we have demonstrated that the $I_{Ca,L}$ of VSMCs was significantly increased after knocking down the expression of Gal-1, it is highly possible that effects of Gal-1 expression on SMC attachment, spreading, and migration might be attributed to the down-regulation of Ca$_{1.2}$ channel surface expression.

Exon 9 is an alternatively splice exon that is 25 amino acids in size and is situated between exons 9 and 10 that code for the α$_{1C}$ I–II loop. Subpopulation of Ca$_{1.2}$ channels containing exon 9 are likely to maintain vasotone due to their unique localization and electrophysiological properties. Here, we report a novel splice-variant specific regulation of Ca$_{1.2}$ channels by Gal-1: that Gal-1 can inhibit the function of Ca$_{1.2,39^+}$ channels, but this inhibition by Gal-1 is abolished by the inclusion of exon 9. Interestingly, in silico computer secondary structure prediction of Ca$_{1.2}$ I–II loop indicates that in the presence of exon 9 the likelihood of forming an α-helix in the ER export signal region is significantly increased (Online Figure V). Whether the affinity of binding of Gal-1 to the ER export signal depends on the secondary structure of this signal will require future work. An alternative explanation could be that the Gal-1 ER export signal
signal, which contains many negatively charged amino resi-
dues, may interact with the positively charged amino acids
found in the neighboring exon 9*, and hence this interaction
abrogates Gal-1 binding and modulation. Such an interaction
between exon 9* and the ER export signal may also affect
surface expression, and we observed that the current density
of exon 9*-containing CaV1.2 channels (CaV1.2b) are signif-
ificantly lower than those that do not contain exon 9*
(CaV1.277WT) (Figure 3B and 3D). Taken together, these
results suggest that the inclusion of exon 9* will prevent the
interaction between Gal-1 and CaV1.2p channels or that the
ER export signal is masked by exon 9* and still allowing
the subpopulation of CaV1.2p channels to continue in its role
to maintain vasotone.8,10 However, exon 9* was found to be
absent in VSMC in the pathological disease, such as athero-
sclerosis.45 Therefore, Gal-1 could be an important modulator
of pathological electric remodeling in blood vessels, and it
would be of interest to examine the level of expression Gal-1
in arteries from atherosclerotic or hypertensive patients or in
any rodent models of hypertension to ascertain whether Gal-1
expression is a cause or a compensatory mechanism in the
pathogenesis of hypertension.

Vascular hypertension has been shown to be tightly asso-
ciated with the enhanced activity of the vascular CaV1.2 calcium
channels. Significantly, the VSMCs of hypertensive rats have increased surface expression of CaV1.2 calcium
channels that contributed to elevated Ca2+ influx and the development of abnormal arterial tone.3,42 Whole-cell elec-
trophysiological recordings of VSMC isolated from SHR rats
showed elevated currents.43,44 Furthermore, single-channel
recordings revealed an increased number of CaV1.2 calcium
channel openings, while single-channel conductance or open-
time distribution remained unchanged.45 Our data showed
that overexpression of Gal-1 can decrease the functional
surface expression of CaV1.2. Conversely, knock-down of
Gal-1 protein level by siRNA increased I_Ca,L in rat A7r5 cells and isolated VSMCs. In rat MAs, the functional outcome
of knock-down of Gal-1 was larger maximal constriction
induced by extracellular K+. These observations suggest
that the absence of Gal-1 in resistance arteries contributes to
higher blood pressure, and indeed Gal-1–/– null mice showed
a greater response in the increase of right ventricular and
pulmonary arterial blood pressure caused by acute hypoxia
compared with wild-type mice.18

Taken together, our data highlight that Gal-1 modulates the
CaV1.2 currents in a splice variant specific manner, and its
dynamic expression influences arterial constrictions. Therefore,
its is reasonable to believe that Gal-1 may play some roles in
hypertension via regulating arterial wall constriction, and due
to its selective expression in the blood vessels, Gal-1 may be a
promising target in the management of vascular hypertension.

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Disclosures
None.

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What Is Known?

- The Ca_{1.2} channels play critical roles in vascular smooth muscle contraction and arterial constriction. Alternative splicing provides a posttranscriptional mechanism for exquisite diversity of Ca_{1.2} function in muscle physiology.
- Galectin-1 (Gal-1) is reported to be involved in the regulation of vascular smooth muscle cells (VSMCs), and play a role in the pathogenesis of pulmonary hypertension.

What New Information Does This Article Contribute?

- Gal-1 binds to the II loop of Ca_{1.2} channels to regulate its expression. Gal-1 modulation of Ca_{1.2} function is influenced by alternative exon 9*. Splice-variant specific inhibition of Ca_{1.2} by Gal-1 provides tissue-selective regulation.
- Gal-1 binds to the endoplasmic reticulum (ER) export signal on the C-terminus of exon 9 to prevent surface expression. However, the presence of exon 9* may increase the likelihood of this region to form an α-helical structure to prevent binding by Gal-1, thus suggesting a plausible explanation for splice-variant specific regulation by Gal-1.
- Overexpression of Gal-1 inhibits, while knock-down of Gal-1 increases, Ca_{1.2} currents, indicating that Gal-1 regulates the function of VSMCs via Ca_{1.2} channels. Down-regulation of Gal-1 increases arterial constriction, suggesting that Gal-1’s regulation in blood vessels may play a role in hypertension.

Novelty and Significance

The Ca_{1.2} calcium channels open on sensing membrane depolarization to form conduits for large and rapid Ca^{2+} influx that leads to muscle contraction. The electrophysiological and pharmacological properties of the channel can be modified via alternative splicing, a posttranscriptional mechanism that could generate subtle modifications in channel structure. The channel function can also be modulated through binding with cytoplasmic proteins. We show that Gal-1 reduces the surface expression of Ca_{1.2} channels that do not contain alternative exon 9*, via the possible masking of the ER export signal found on the C-terminal portion of exon 9. The presence of exon 9* was predicted by in silico protein structure software to induce the formation of an α-helical structure in the ER export signal to either prevent the binding of Gal-1 or interact with exon 9* to mask the ER export signal. Importantly, down-regulation of Gal-1 increases Ca_{1.2} channel expression and conductivity of mesenteric arteries. As Gal-1 is expressed in smooth and not cardiac muscles, it may potentially be a therapeutic target to the pathogenesis of hypertension.
Splice Variant Specific Modulation of Cav1.2 Calcium Channel by Galectin-1 Regulates Arterial Constriction

Juejin Wang, Sharon S.C. Thio, Sophia S.H. Yang, Dejie Yu, Chye Yun Yu, Yuk Peng Wong, Ping Liao, Shengnan Li and Tuck Wah Soong

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SUPPLEMENTAL MATERIAL

Methods

Human Tissues and Animals
The human arteries were obtained from patients who accepted coronary artery bypass surgery (Dept. of Internal Medicine, Singapore General Hospital, Singapore), and the experiments were confirmed to the principles outlined in the Declaration of Helsinki. Adult male Sprague-Dawley (SD) rats (8-10 weeks old) were maintained on standard chow and tap water ad libitum. Animals were euthanized by CO₂, a midline incision was made to expose the thoracic and abdominal cavities, and then aorta and mesentery were quickly collected for isolation of VSMCs and MAs. All animal work has been approved by and done in accordance with the Institutional Animal Care and Use Committee (IACUC) guidelines of the National University of Singapore.

Isolation of Rat VSMCs
Rat VSMCs were freshly isolated from rat aortas. Briefly, excised aortas were placed in a petri dish containing hanks' balanced salt solution (HBSS) and excess fat was removed under a dissecting microscope. Aortas were cut longitudinally and placed in digestion flasks with type II collagenase (Sigma, 175U/ml) and elastase (Sigma, 1.25U/ml) for 20 min at 37 °C in a shaker bath. The adventitia was removed and aortas were cut into 1- to 2-mm segments, incubated with type II collagenase (175U/ml) and elastase (2.5U/ml) in HBSS for 1-2 h at 37 °C until full dispersion into single-cell suspension was achieved. Cells were centrifuged at 900 rpm for 5 min, and the pellet was collected. Cells were resuspended in 5-7 ml DMEM with 20% FBS and transferred to 10 mm flask. Next day, the cells were changed with DMEM with 10% FBS and 1% penicillin and streptomycin. Only the first generation of isolated VSMCs was used for experiments.

DNA Plasmids
We performed PCR from human aorta cDNA library (Clontech, catalog No. #HL4040AH) to amplify human Gal-1. The Gal-1 PCR product was isolated and sub-cloned into pGEM-T Easy vector (Promega) and its identity was confirmed by automated DNA sequencing analysis (NCBI NM_002305.3). Following, the plasmids was digested by EcoRI enzyme, and then the Gal-1 fragment was inserted into pIRE52-DsRed expression vector (Clontech). Human α1c subunits (CaV1.277WT, CaV1.2SM, CaV1.2b and CaV1.2CM), β2a subunits and α2δ subunits used here have been reported in our previous work.1-3 For GST constructs, different regions of CaV1.2 I-II loop were amplified by PCR and the PCR products were ligated into pGEM-T Easy vector. After validation by DNA sequencing, the plasmids were isolated by restriction enzyme digestions and, sub-cloned into the pGEX4T-1 vector (GE Healthcare) to generate the various GST fusion constructs.

RT-PCR
Total RNA was extracted from cells or tissues with Trizol reagent (Invitrogen) following the manufacturer’s protocol, and then 1 µg of total RNA was reverse transcribed to cDNA using SuperScript III reverse transcriptase (Invitrogen). PCR was performed with primers as listed in Online Table I.

Isolation of Cell Membrane Proteins
All membrane preparation steps were carried out at 4 °C or on ice. Transfected cells were scraped off from plates and washed in ice-cold PBS. Cells were resuspended in ice-cold lysis buffer (20 mM
Tris-HCl, 150 mM NaCl, pH 7.4, protease inhibitor added), sonicated by low power ultrasound for 1 min, incubated for 15 min on ice, and gently resuspended through a pipette tip every 3-5 min. The lysate was centrifuged for 10 min at 1000 g. Membranes in the supernatant were collected, and centrifuged at 90,000 g for 40 min. The supernatant was collected as cytosol protein, and the final pellet was resuspended in lysis buffer as membrane protein for later analysis.

**Western Blotting**

Total protein was extracted from VSMCs or arterial tissues in lysis buffer, and the protein concentration was determined by Bradford assay (Bio-Rad). Protein samples were boiled at 95 °C in 6× SDS-sample buffer for 5 min; equal amount protein samples were separated by SDS-PAGE, and then electrically transferred to PVDF membranes (Millipore). After blocking, PVDF membranes were incubated with primary antibodies overnight at 4 °C or 1 h at room temperature, followed by incubation with HRP-linked secondary antibody (Sigma, 1:5000 dilutions) 1 h at room temperature. Blots were visualized with enhanced chemiluminescence reagent (Pierce) before exposing to an X-ray film. The relative band density was analyzed by Quantity One software (Bio-Rad).

**Immunofluorescence**

The colocalization of CaV1.2 and Gal-1 in rat MAs was determined by immunofluorescence staining. In brief, fresh isolated MAs were fixed in 4% PFA in PBS 2-4 h at 4 °C, then leave tissue in 20-30% sucrose in PBS overnight. The tissues were cryosectioned to 20 μm in thickness, the sections were blocked with 0.3% BSA and 0.1% Triton in PBS for 40 min, and then incubated with rabbit anti-CaV1.2 pan α1C (Alomone, 1:100 dilutions), mouse anti-α-SM actin (Millipore, 1:100 dilutions) or goat anti-Gal-1 (Santa Cruz, 1:100 dilutions) antibodies dissolved in blocking solution at 4 °C overnight, immunofluorescent control was performed by replacing the primary antibodies with blocking solution. After washing with 0.1% Triton/PBS, the sections were incubated with the secondary antibodies labeled with FITC or Cy3 at room temperature for 1 h. The immunostainings were visualized using a laser-scanning confocal microscope (Fluoview BX61; Olympus).

**Cell Culture and Transfection**

Human embryonic kidney 293 (HEK 293) cells and rat VSMCs were cultured in DMEM medium (Invitrogen) supplemented with 10% FBS and 1% penicillin and streptomycin and maintained in 5% CO2 incubator at 37 °C. For transfection, cells were seeded on the petri dishes containing cover slips and grown overnight, human CaV1.2 calcium channels were cotransfected with Gal-1 in HEK 293 cells by a calcium-phosphate mediated method. In the 35 mm petri dish, 0.5 μg plasmids of human Gal-1 and 1.7 μg human CaV1.2 (CaV1.2WT (1-8-Δ9*-33), CaV1.2SM (1-8-9*-Δ33), CaV1.2b (1-8-9*-33) or CaV1.2CM (1a-8a-Δ9*-33)) together with 1.25 μg of human β2a, 1.25 μg of α2δ, and 0.25 μg of TAG (T antigen) were transiently cotransfected into HEK 293 cells. siRNA targeting with Gal-1 (DharmaFECT® Thermo Scientific) were transfected into rat A7r5 cells and isolated VSMCs using Lipofectamine 2000 kit (Invitrogen) following the manufacturer’s protocol. Transfected HEK 293 cells and VSMCs were grown for 36-48 h in 5% CO2 incubator at 37 °C before patch clamp analysis.

**Whole-cell Patch Clamp Recordings**

The external solution for recording HEK 293 cells contained (in mM) 144 or 140 TEA-MeSO3, 10 HEPES, 1.8 CaCl2 or 5 BaCl2, pH was adjusted to 7.4 with CsOH and osmolarity to 300-310 mOsm with glucose). The internal solution (pipette solution) contained (in mM) 138 Cs-MeSO3, 5 CsCl, 0.5 EGTA, 10 HEPES, 1 MgCl2, 2 mg/ml Mg-ATP, pH 7.3 (adjusted with CsOH). Glucose was used to
adjust the osmolarities of solutions to between 290 and 300 mOsm. The external solution for recording VSMCs contained (in mM) 132 TEA-Cl, 10 HEPES, 1 MgCl₂, 10 BaCl₂, 10 glucose, pH 7.4, osmolarities 300-310 mOsm. The internal solution (pipette solution) contained (in mM) 130 CsCl, 5 EGTA, 1 MgCl₂, 10 HEPES, 2 Na₂ATP, 0.5 GTP, 10 glucose, pH 7.2, osmolarities 290-300 mOsm. Whole cell currents obtained under voltage clamp with an Axopatch 200B amplifier (Molecular Device), were filtered at 1-5 kHz and sampled at 5-50 kHz, and the series resistance was typically < 5 MΩ after > 70% compensation. The P/4 protocol was used to subtract online the leak and capacitative transients.

The current-voltage (I-V) relationship, steady-state activation, steady-state inactivation, recovery from inactivation recording and the data analysis were performed as described in our previous studies. To determine the whole cell current-voltage (I-V) relationships, currents were recorded by holding the cell at -90 mV before stepping to various potentials from -50 to 50 mV over 900 ms. The I-V curve was fitted with the equation: $I_{Ba} = G_{max}(V - E_{rev})/(1 + \exp((V - V_{1/2})/k)$, where $G_{max}$ is the maximum conductance; $E_{rev}$ is the reversal potential; $V_{1/2}$ is the half-activation potential; and $k$ is the slope. G-V curves were obtained from a tail activation protocol. The cells were activated by a 6 ms test pulse of variable voltage family from -70 to 100 mV, and tail currents were measured after repolarization to -50 mV for 10 ms. The tail currents were normalized to the peak currents before fitting with a dual Boltzmann equation: $G/G_{max} = F_{low}/(1 + \exp((V_{1/2,low} - V)/k_{low}) + (1-F_{low})/(1 + \exp((V_{1/2,high} - V)/k_{high}))$, where $G$ is the tail current and $G_{max}$ is the peak tail current, $F_{low}$ is the fraction of low threshold component; $V_{1/2,low}$, $k_{low}$, and $k_{high}$ are the half-activation potentials and slope factors for the low and high threshold components. The steady-state inactivation curves were obtained from experiments by stepping from a holding potential of -90 mV to a 30 ms normalizing pulse to 10 mV followed by a family of 5 or 15 s long prepulses from -100 to 20 mV. A 104 ms test pulse to 10 mV was recorded after giving a 3 ms pulse at -90 mV finally. Each test pulse was normalized to the maximal current amplitude of the normalizing pulse. The steady-state inactivation data were fitted with a single Boltzmann equation: $I_{relative} = I_{min} + (I_{max} - I_{min})/(1 + \exp((V_{1/2} - V)/k)$, where $I_{relative}$ is the normalized current; $V_{1/2}$ is the potential for half-inactivation, and $k$ is the slope value. Recovery from inactivation of $I_{Ca}$ or $I_{Ba}$ was investigated using a standard two-pulse protocol. Fractional recovery of peak $I$ were plotted against $\Delta T$. Inactivation time constants ($\tau$) were determined from the double exponential equation: $Y = Y_{min} + A1 \times [1 - \exp(-t/\tau_f)] + A2 \times [1 - \exp(-t/\tau_s)]$, where $Y$ is the fraction of recovery, $A1$ and $A2$ are the maximum values of the fast and slow component, and $\tau_f$ and $\tau_s$ are the time constants, respectively. ON-gating charge ($Q_{ON}$) was measured by holding the cells at -90 mV before applying a 6 ms long pulse at $V_{rev}$ where no ionic inward and outward currents were observed. $Q_{ON}$ currents were quantified by current integration over the first 2 ms of the test pulse to $V_{rev}$, and normalized to cell capacitance.

The Introduction of siRNA into Rat MAs
MAs were dissected from 8-10 weeks old male SD rats. The introduction of siRNA into rat MAs was achieved by a reversible permeabilization (RP) procedure. Briefly, arterial segments were first incubated at 4 °C for 20 min in the following solution (in mmol/L): 120 KCl, 2 MgCl₂, 10 EGTA, 5 ATP, and 20 TES (pH 6.8). Arteries were then incubated in a similar solution containing 40 nmol/L siRNA, 120 KCl, 2 MgCl₂, 5 ATP, and 20 TES (pH 6.8) for 3 h at 4 °C and then in a solution containing elevated 10 MgCl₂, 40 nmol/L siRNA, 120 KCl, 5 ATP, and 20 TES (pH 6.8) for 30 min. Permeabilization of arteries was reversed by incubating arteries for 30 min at room temperature in physiological solution containing the following (in mmol/L): 140 NaCl, 5 KCl, 10 MgCl₂, 5 glucose,
and 2 MOPS (pH 7.1). [Ca\(^{2+}\)] was next gradually increased in this solution from nominally Ca\(^{2+}\)-free to 0.01, 0.1, and 1.8 mmol/L over a period of 45 min. Following RP, arterial segments were organ cultured by placing the arteries in serum-free DMEM-F12 culture media (Invitrogen) and incubating at 37 °C and 5% CO\(_2\) for 1 to 4 days. After 1-4 days, the arteries were collected for western blot to check the endogenous expression of Gal-1.

**Diameter Recording in Pressurized Vessels**

Cultured mesenteric arterial segments were cannulated on glass micropipettes mounted in a 5 ml myograph chamber (Living System, University of Vermont Instrumentation and Model Facility) as described previously.\(^9,\(^10\) Only secondary branches of MA were used in this experiment. Following cannulation, arteries were pressurized at 20 mmHg and continuously superfused with aerated physiological saline solution (PSS) at 37 °C and pH 7.4 for 30 min to allow equilibration. Arterial diameter was measured with video edge detection equipment and recorded using data acquisition software (Dataq Instruments, Akron, OH). Then, arteries were exposed to PSS containing elevated [K\(^+\)], made by isoosmotic replacement of NaCl with KCl. Arterial constriction was expressed using the following equation: \%

\[
\text{%Constriction} = \left[1 - \left(\frac{D - D_{\text{min}}}{D_{\text{max}} - D_{\text{min}}}\right)\right] \times 100,
\]

where \(D_{\text{max}}\) is the maximum diameter obtained in Ca\(^{2+}\)-free PSS containing diltiazem (Sigma, 100 µmol/L) and forskolin (Sigma, 1 µmol/L) and \(D_{\text{min}}\) is the minimum diameter obtained with the Ca\(^{2+}\) ionophore ionomycin (Sigma, 10 µmol/L) at the end of each experiment. Ionomycin-induced constrictions are presented as a percentage of maximum diameters (\(D_{\text{max}}\)). Arteries not achieving > 70% constriction in response to ionomycin were not used for analysis. Half-maximal effective concentration (EC\(_{50}\)) was determined from each [K\(^+\)]\(_o\) concentration-response experiment.
References


### Oligonucleotide primers (5’-3’)

<table>
<thead>
<tr>
<th>Oligonucleotide primers (5’-3’)</th>
<th>Product length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human Gal-1 Forward: ATGGCTTGTGGTCTGGTCGCC; Reverse: TCAGTCAAAGGCCACACATT</td>
<td>408</td>
</tr>
<tr>
<td>Human CaV1.2 Forward: GAGTTTTCCAAAGAGAGG; Reverse: GCTGAACTTTGACTTGGAGATCC</td>
<td>489 (9*)</td>
</tr>
<tr>
<td>Rat Gal-1 Forward: ATGGCCCTGTGGTCTGGT; Reverse: TCACTCAAAGGCCACACACATT</td>
<td>408</td>
</tr>
<tr>
<td>Rat CaV1.2 Forward: CCGTGTGCAAAGCCGGGTTGG; Reverse: GGATTTGGAGATCCGGTGG</td>
<td>587(9*)</td>
</tr>
<tr>
<td>Human &amp; rat GAPDH Forward: CTTCACCACCATGGAGAAGG; Reverse: CTTACTCCTTGGAGGCCATG</td>
<td>707</td>
</tr>
</tbody>
</table>

**Online Table I. Primer sequences for PCR.**

### Summary of steady-state activation and inactivation parameters in different CaV1.2 isoforms.

<table>
<thead>
<tr>
<th>CaV1.2 isoforms</th>
<th>V1/2,act (mV)</th>
<th>V1/2,inact (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ctrl n Gal-1</td>
<td>Ctrl n Gal-1 n</td>
</tr>
<tr>
<td>CaV1.2&lt;sub&gt;77WT&lt;/sub&gt;</td>
<td>13.09±0.88 11 10.56±0.84 11</td>
<td>-31.39±0.68 5 -31.20±0.79 6</td>
</tr>
<tr>
<td>CaV1.2&lt;sub&gt;SM&lt;/sub&gt;</td>
<td>12.48±0.83 10 9.96±0.96 7</td>
<td>-31.09±0.81 5 -30.42±0.78 6</td>
</tr>
<tr>
<td>CaV1.2&lt;sub&gt;b&lt;/sub&gt;</td>
<td>6.81±0.73 8 7.87±0.77 12</td>
<td>-32.43±0.44 8 -31.03±0.47 6</td>
</tr>
<tr>
<td>CaV1.2&lt;sub&gt;CM&lt;/sub&gt;</td>
<td>9.33±1.13 11 9.64±1.20 7</td>
<td>-31.24±0.62 8 -22.78±0.57** 9</td>
</tr>
</tbody>
</table>

**Online Table II. Summary of steady-state activation and inactivation parameters in different CaV1.2 isoforms.** V1/2,act: half-activation potentials, V1/2,inact: half-inactivation potentials. **, p < 0.01 vs. control, unpaired t test.

### Summary of recovery from inactivation parameters in different CaV1.2 isoforms.

<table>
<thead>
<tr>
<th>CaV1.2 isoforms</th>
<th>τ fast (ms)</th>
<th>τ slow (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ctrl n Gal-1</td>
<td>Ctrl n Gal-1</td>
</tr>
<tr>
<td>CaV1.2&lt;sub&gt;77WT&lt;/sub&gt;</td>
<td>276.4±22.6 7 495.6±29.4 7</td>
<td>5.37±1.29 7 5.96±1.99 7</td>
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<tr>
<td>CaV1.2&lt;sub&gt;SM&lt;/sub&gt;</td>
<td>71.8±102.9 5 N.D.</td>
<td>5.40±1.30 5 4.85±1.83 5</td>
</tr>
<tr>
<td>CaV1.2&lt;sub&gt;b&lt;/sub&gt;</td>
<td>N.D.</td>
<td>357.4±190.7 5 3.44±0.53 5 5.72±1.72 5</td>
</tr>
<tr>
<td>CaV1.2&lt;sub&gt;CM&lt;/sub&gt;</td>
<td>N.D.</td>
<td>326.2±266.9 6 4.12±0.91 6 6.71±2.25 6</td>
</tr>
</tbody>
</table>

**Online Table III. Summary of recovery from inactivation parameters in different CaV1.2 isoforms.**

### Summary of electrophysiological properties in A7r5 cell when transfected with NT siRNA or Gal-1 siRNA.

<table>
<thead>
<tr>
<th></th>
<th>V1/2,inact (mV)</th>
<th>n</th>
<th>τ fast (ms)</th>
<th>n</th>
<th>τ slow (s)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>NT siRNA</td>
<td>-31.71±1.05</td>
<td>5</td>
<td>719.4±304.4</td>
<td>9</td>
<td>11.10±7.55</td>
<td>9</td>
</tr>
<tr>
<td>Gal-1 siRNA</td>
<td>-30.20±0.81</td>
<td>8</td>
<td>465.0±324.1</td>
<td>6</td>
<td>6.61±2.11</td>
<td>6</td>
</tr>
</tbody>
</table>

**Online Table IV. Summary of electrophysiological properties in A7r5 cell when transfected with NT siRNA or Gal-1 siRNA.** V1/2,inact: half-inactivation potentials.
### Online Table V. Diameter values for [K\(^+\)]\(_o\) concentration-response experiments

Diameter values were shown for NT siRNA and Gal-1 siRNA treated arteries in PSS of 6 mmol/L extracellular K\(^+\) [K\(^-\)]\(_o\), 80 mmol/L [K\(^-\)]\(_o\), and 120 mmol/L [K\(^-\)]\(_o\) containing 10 µmol/L ionomycin. Ionomycin induced constriction, expressed as percent decrease from maximum diameter, and EC\(_{50}\) values, calculated from [K\(^-\)]\(_o\) concentration response curves, were also shown. *, p < 0.05 vs. NT siRNA treated arteries, unpaired t test.

<table>
<thead>
<tr>
<th></th>
<th>NT siRNA</th>
<th>Gal-1 siRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Day 1</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diameter, µm</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6 mmol/L [K(^-)](_o)</td>
<td>237±5.5</td>
<td>283±30.7</td>
</tr>
<tr>
<td>80 mmol/L [K(^-)](_o)</td>
<td>60±7.6</td>
<td>61±8.5</td>
</tr>
<tr>
<td>120 mmol/L [K(^-)](_o) + Ionomycin</td>
<td>35±5.7</td>
<td>32±2.4</td>
</tr>
<tr>
<td>Ionomycin induced constriction, % of maximum diameter</td>
<td>82±2.3</td>
<td>77±5.2</td>
</tr>
<tr>
<td>[K(^-)](<em>o) EC(</em>{50})</td>
<td>33±0.5</td>
<td>31±1.2</td>
</tr>
<tr>
<td><strong>Day 2</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diameter, µm</td>
<td></td>
<td></td>
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<tr>
<td>6 mmol/L [K(^-)](_o)</td>
<td>239±9.8</td>
<td>283±13.7</td>
</tr>
<tr>
<td>80 mmol/L [K(^-)](_o)</td>
<td>50±11.9</td>
<td>58±1.7</td>
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<td>120 mmol/L [K(^-)](_o) + Ionomycin</td>
<td>39±15.3</td>
<td>43±3.2</td>
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<tr>
<td>Ionomycin induced constriction, % of maximum diameter</td>
<td>82±5.4</td>
<td>81±1.2</td>
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<tr>
<td>[K(^-)](<em>o) EC(</em>{50})</td>
<td>29±0.9</td>
<td>31±0.7</td>
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<tr>
<td><strong>Day 3</strong></td>
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<td>Diameter, µm</td>
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<tr>
<td>6 mmol/L [K(^-)](_o)</td>
<td>266±14.4</td>
<td>239±17.3</td>
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<td>80 mmol/L [K(^-)](_o)</td>
<td>81±16.8</td>
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<td>120 mmol/L [K(^-)](_o) + Ionomycin</td>
<td>52±13.0</td>
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<td>Ionomycin induced constriction, % of maximum diameter</td>
<td>79±4.4</td>
<td>85±2.8</td>
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<td>[K(^-)](<em>o) EC(</em>{50})</td>
<td>34±1.2</td>
<td>33±0.9</td>
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<tr>
<td><strong>Day 4</strong></td>
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</tr>
<tr>
<td>Diameter, µm</td>
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<td></td>
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<tr>
<td>6 mmol/L [K(^-)](_o)</td>
<td>257±10.1</td>
<td>263±9.3</td>
</tr>
<tr>
<td>80 mmol/L [K(^-)](_o)</td>
<td>104±10.4</td>
<td>58±11.2*</td>
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<tr>
<td>120 mmol/L [K(^-)](_o) + Ionomycin</td>
<td>63±7.9</td>
<td>46±8.3</td>
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<tr>
<td>Ionomycin induced constriction, % of maximum diameter</td>
<td>77±2.7</td>
<td>83±2.0</td>
</tr>
<tr>
<td>[K(^-)](<em>o) EC(</em>{50})</td>
<td>37±4.5</td>
<td>33±2.0</td>
</tr>
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</table>
Online Figure I. Coomassie blue staining of GST fusion proteins. A, Coomassie blue staining of the negative control (upper panel) showed little GST fusion proteins when no IPTG was used. While it showed robust expression of various GST fusion proteins of Ca\textsubscript{v}1.2 I-II loop induced by IPTG (middle panel). B, High expression of exon 9 truncated constructs after IPTG induction. C, High expression of exon 9 deletional proteins after IPTG induction.
Online Figure II. The effects of Gal-1 on the channel kinetics of the CaV1.2\textsubscript{77WT}, CaV1.2\textsubscript{SM}, CaV1.2\textsubscript{b} and CaV1.2\textsubscript{CM}. A, Plots of steady-state activation, steady-state inactivation and recovery from inactivation curves of CaV1.2\textsubscript{77WT} cotransfected with vector (black) or Gal-1 (gray) derived from recordings in bath solution containing 1.8 mmol/L Ca\textsuperscript{2+}. Steady-state activation and inactivation and recovery from inactivation curves of CaV1.2\textsubscript{SM} (B), CaV1.2\textsubscript{b} (C) and CaV1.2\textsubscript{CM} (D) were also measured in 1.8 mmol/L Ca\textsuperscript{2+} bath solution, respectively.
Online Figure III. Gal-1 can decrease the surface membrane expression of CaV1.2 α1C subunits. A, The membrane surface protein was isolated from HEK 293 cells which were cotransfected with CaV1.2α77WT +/- Gal-1. Approximately 100 µg membrane proteins were separated in 6% SDS gel, and immunolabeled with anti-CaV1.2 pan-α1C antibody (Alomone Labs, 1:1000 dilutions), and anti-calnexin antibody (Santa Cruz, 1:1000 dilutions) as loading control. B, The relative band density was analyzed in control (black, n = 3) or Gal-1 (gray, n = 3) cotransfected HEK 293 cells. *, p < 0.05 vs. control, unpaired t test.
Online Figure IV. Gal-1 dose-dependently inhibits $I_{Ca,L}$ of CaV1.2<sup>77WT</sup>. A, Different amounts of Gal-1 were cotransfected with constant amounts of β2a and CaV1.2<sup>77WT</sup> subunits, then $I_{Ca,L}$ was recorded by Tail protocol in cotransfected HEK 293 cells in 1.8 mmol/L Ca<sup>2+</sup> external solution. B, The relative current inhibition was analyzed from the peak Tail currents when holding at 100 mV. *, $p < 0.05$ vs. control, one-way ANOVA followed by Bonferroni post hoc test.
Online Figure V. The secondary structure prediction of CaV1.2 I-II loop. The secondary structure was predicted by Jpred 3 Secondary Structure Prediction Server (http://www.compbio.dundee.ac.uk/www-jpred/). The absence of exon 9* affects the C-terminus of exon 9 next to the AID domain (highlight with blue) to form helix structure. The red sequences indicated di-basic motifs. H, helix structure. Jnet, final secondary structure prediction for query. Jnet Rel, Jnet reliability of prediction accuracy, ranges from 0 to 9, the larger of the number, the better is the confidence of the prediction.
Online Figure VI. Knock-down the expression of Gal-1 does not change calcium channel properties of A7r5 cells. A, The endogenous expression of Gal-1 was detected by western blot after treatment with siRNAs targeting on the different regions of Gal-1 mRNA. α-SM actin was detected as internal control. B, Plots of steady-state inactivation treated with NT siRNA (black, n = 5) or Gal-1 siRNA (gray, n = 8) derived from recordings in bath solution containing 10 mmol/L Ba^{2+}. C, Recovery from inactivation of $I_{Ba}$ was investigated after NT siRNA (black, n = 9) or Gal-1 siRNA (gray, n = 6) treatment using a standard two-pulse protocol when 10 mmol/L Ba^{2+} was used as charger carrier. Fractional recovery of peak $I_{Ba}$ was plotted against $\Delta T$. 
Online Figure VII. L-type calcium channel blocker nifedipine completely blocks the barium currents of A7r5 cells. A, The effect of nifedipine (Nif) with two concentrations 1 µmol/L (black, n = 9) and 10 µmol/L (gray, n = 5) on the currents of A7r5 cells in 10 mmol/L Ba\(^{2+}\) bath solution. The peak \(I_{Ba}\) was recorded by single pulse protocol. \(p = 0.5265\), unpaired \(t\) test. B, \(I_{Ba}\) was measured in NT siRNA (black, n = 8) or Gal-1 siRNA (gray, n = 9) treated A7r5 cells before and after application of Nif (1 µmol/L) in 10 mmol/L Ba\(^{2+}\) bath solution. *, \(p < 0.05\) vs. NT siRNA treated cells, unpaired \(t\) test.
Online Figure VIII. Knock-down of Gal-1 increases arterial constriction induced by $[\text{K}^+]_o$. Concentration-response curves of $[\text{K}^+]_o$ for NT siRNA (black) or Gal-1 siRNA (gray) effects on the constriction of mesenteric arteries at day 1, day 2 and day 4. *, $p < 0.05$; **, $p < 0.01$ vs. NT siRNA treated MAs, unpaired $t$ test.