Native smooth muscle L-type Ca_{1.2} calcium channels have been shown to support a fraction of Ca^{2+} currents with a window current that is close to resting potential. The smooth muscle L-type Ca^{2+} channels are also more susceptible to inhibition by dihydropyridines (DHPs) than the cardiac channels. It was hypothesized that smooth muscle Ca_{1.2} channels exhibiting hyperpolarized shift in steady-state inactivation would contribute to larger inhibition by DHP, in addition to structural differences of the channels generated by alternative splicing that modulate DHP sensitivities. In addition, it has also been shown that alternative splicing modulates DHP sensitivities by generating structural differences in the Ca_{1.2} channels. Here, we report a smooth muscle L-type Ca_{1.2} calcium channel splice variant, Ca_{1.2}SM (1/8/9*/32/33), that when expressed in HEK 293 cells display hyperpolarized shifts for steady-state inactivation and activation potentials when compared with the established Ca_{1.2}b clone (1/8/9*/32/33). This variant activates from more negative potentials and generates a window current closer to resting membrane potential. We also identified the predominant cardiac isoform Ca_{1.2}CM clone (1a/8a/Δ9*32/33) that is different from the established Ca_{1.2}a (1a/8a/Δ9*/31/33). Importantly, Ca_{1.2}SM channels were shown to be more sensitive to nifedipine blockade than Ca_{1.2}b and cardiac Ca_{1.2}CM channels when currents were recorded in either 5 mM Ba^{2+} or 1.8 mM Ca^{2+} external solutions. This is the first time that a smooth muscle Ca_{1.2} splice variant has been identified functionally to possess biophysical property that can be linked to enhanced state-dependent block by DHP.

L-type voltage-gated Ca_{1.2} calcium channels are essential for muscle excitation-contraction coupling and, in particular, for vasotone in smooth muscles (1). It has been hypothesized that a subpopulation of smooth muscle Ca_{1.2} calcium channels that activate and inactivate close to resting potentials will underlie basal contractility to contribute to vasotone (2–4). However, this subpopulation of Ca_{1.2} calcium channels has not been identified. Of the four subunits (α_1, β, α_2/δ, and γ) that composed the calcium channels, the α_1-subunit not only forms the aqueous pore but is also the site for binding of organic agonists or antagonists (5, 6). The Ca_{1.2}L型 calcium channels are localized mainly in the brain, cardiac, and smooth muscles and a large number of alternative splicing sites of the α_1-subunit have been reported in various tissues (7–9). Changes in the expression levels or mutations of alternatively spliced exons of the Ca_{1.2} calcium channel have also been identified in development, heart failure, myocardial infarction, and congenital heart disease (10–14). Importantly, alternative splicing serves to diversify Ca_{1.2} calcium channel biophysical properties and as such it is reasonable to test our first hypothesis as to whether a Ca_{1.2} splice variant may possess properties that are more similar to the subpopulation of channels that exhibit hyperpolarized window current.

L-type calcium channel blockers, 1,4-dihydropyridines (DHPs), 2 phenylalkylamines, and benzothiazepines, are being used in the management of cardiovascular diseases (5, 15, 16). Two mechanisms have been suggested for the observed vascular selectivity of DHP inhibition over cardiac Ca_{1.2} channels. One mechanism was the more depolarized resting membrane potential of smooth muscles (17) and the second was the difference in voltage dependence of DHP modulation arising from tissue-specific alternative splicing of mutually exclusive exons 8 and 8a that code for IS6 in smooth and cardiac muscle Ca_{1.2} channels (18). Characterization of cDNA clones of Ca_{1.2} channels isolated from smooth and cardiac muscles segregated two clones as the cardiac muscle form Ca_{1.2}a (α_{1Ca}), channel and smooth muscle form Ca_{1.2}b (α_{1CM}) channel (19, 20). Both isoforms were reported to have minor differences at four alternative splicing loci amounting to ~5% of their amino acid sequence. The four splice sites are: exon 1/1 in the N terminus, exon 8/8a in the transmembrane segment IS6, exon 31/32 in the transmembrane segment IVS3, and exon 9 in the cytoplasmic loop connecting domains I and II. Ca_{1.2}a was characterized to contain alternatively spliced exons 1a/8a/Δ9*/31/33 (20), whereas Ca_{1.2}b contained alternatively spliced exons 1/8/9*/32/33 (19). Notably, both channels share similar activation and...
inactivation properties (21, 22). However, Ca\textsubscript{\textit{v}}\textsubscript{1.2}b channels are more sensitive to nisoldipine due to the presence of the alternatively spliced exon 8 within the IS6 segment (18, 21). Importantly, these previous works reported the lack of linkage between the gating properties of Ca\textsubscript{\textit{v}}\textsubscript{1.2} smooth and cardiac muscle splice variants with vascular specificity for DHP inhibition (18, 21). We have previously shown by systematic transcript scanning that the Ca\textsubscript{\textit{v}}\textsubscript{1.2} subunit is subject to extensive alternative splicing generating potentially a large number of splice combinations (8). For our second hypothesis, we tested whether a smooth muscle Ca\textsubscript{\textit{v}}\textsubscript{1.2} splice variant exists that possesses a gating property that will link its steady-state inactivation property to the potency of inhibition by DHP as blockade by DHP is state-dependent.

In the present study, we report a smooth muscle Ca\textsubscript{\textit{v}}\textsubscript{1.2} splice variant (Ca\textsubscript{\textit{v}}\textsubscript{1.2}SM) that differs from the notable smooth muscle Ca\textsubscript{\textit{v}}\textsubscript{1.2}b channel that lacks exon 33. Here we showed that the Ca\textsubscript{\textit{v}}\textsubscript{1.2}SM calcium channels exhibited a more hyperpolarized Ca\textsuperscript{2+} window current that supports our first hypothesis. Interestingly, the same Ca\textsubscript{\textit{v}}\textsubscript{1.2}SM splice variant also possesses electrophysiological and pharmacological properties that support our second hypothesis to link altered biophysical property of a smooth muscle Ca\textsubscript{\textit{v}}\textsubscript{1.2} channel splice variant to the potency of DHP inhibition. Taken together, we showed that unexpectedly a single smooth muscle Ca\textsubscript{\textit{v}}\textsubscript{1.2} splice variant, Ca\textsubscript{\textit{v}}\textsubscript{1.2}SM, underlies both a hyperpolarized window current that is reminiscent of the window current of a subpopulation of channels observed in smooth muscles (2–4) and a more potent inhibition by nifedipine in correlation with a hyperpolarized shift in steady-state inactivation potentials.

**EXPERIMENTAL PROCEDURES**

**Generation of Cardiac and Smooth Muscle Isoform of Ca\textsubscript{\textit{v}}\textsubscript{1.2} Calcium Channels**—The construction of human 77 (\textit{D33}) has been described previously (8). It contains the combination of exons 1/8/9\textsuperscript{\textit{a}}/32/33. The smooth muscle form (Ca\textsubscript{\textit{v}}\textsubscript{1.2}SM) was generated by substituting a Clal and SgrA1 fragment containing human exon 9\textit{a} into 77 (\textit{D33}), thus generating the splice variant Ca\textsubscript{\textit{v}}\textsubscript{1.2}SM containing alternatively spliced exons 1/8/9\textsuperscript{\textit{a}}/32/33. The human Ca\textsubscript{\textit{v}}\textsubscript{1.2}b contained exons 1/8/9\textsuperscript{\textit{a}}/32/33 (18, 19, 21, 23) and was generated previously (24). To generate the construct of the cardiac muscle form (Ca\textsubscript{\textit{v}}\textsubscript{1.2}CM), a two-step cloning was performed. A fragment containing human exon 8a was digested with BstA1 and Clal and cloned into the human 77 (WT) construct provided by Dr. Roger Zuhlke to replace exon 8. Human exon 1a was further introduced to this construct using HindIII and AsfI restriction enzymes. Thus the Ca\textsubscript{\textit{v}}\textsubscript{1.2}CM construct contains exons 1a/8a/9\textsuperscript{\textit{a}}/32/33.

**RT-PCR, Colony Screening, and Restriction Enzyme Digestion**—Young adult male Wistar rats (150–200 g) were sacrificed by CO\textsubscript{2} followed by subsequent cervical dislocation. All animal experimentations were conducted according to IACUC guidelines and all procedures have been approved by the University’s Animal Ethics Committee. Human radial arteries were obtained, with informed consent, from patients who underwent coronary artery bypass operations in the National Heart Center of Singapore. The work was approved by the IRB committees of the National Heart Center of Singapore, Singapore General Hospital, and the National University of Singapore.

Total RNA from thoracic aorta, cerebral basilar arteries, mesentery arteries, and hearts were extracted with RNAeasy kits (Qiagen). Reverse transcription for first strand synthesis was carried out using the SUPERSCRIPT™ II RNase H Reverse Transcriptase (Invitrogen) and 18-mer oligo(dT) primer. The oligonucleotide primers used for amplifying rat Ca\textsubscript{\textit{v}}\textsubscript{1.2} ampli
cfimming flanking exon 33 were: GCCCTTCCAGCGTGAGGAG (forward) and TCCCAATCTGCTGATAGATAA (reverse). Primers used to amplify similar regions in human radial arteries were: AACACATCTGCGGCACTG (forward) and AGGTCTGAAGGTGTGTTCCGG (reverse). The PCR protocol includes a denaturation step at 95 °C for 5 min; 35 cycles of 95 °C for 30 s, 52 °C for 45 s, and 72 °C for 1 min; and a final extension step at 72 °C for 10 min. After electrophoresis in 2% agarose gel, the PCR products were excised, extracted, and purified with a Qiagen kit. The PCR amplicons were further cloned into pGEM-T Easy vector (Promega) and transformed into DH10B Escherichia coli cells. White colonies were selected and grown in 96-well plates. Colony PCR with the same set of primers and conditions were performed to identify the presence or absence of exon 33 in each clone. Restriction enzyme digestion was used to investigate the expression of mutually exclusive exons 31 and 32, which have similar size. NsiI digestion was carried out in a 30–μl reaction volume at 37 °C for 5 h.

**Whole Cell Electrophysiological Recordings and Data Analysis**—HEK 293 cells in 35-mm dishes were transiently transfected with Ca\textsubscript{\textit{v}}\textsubscript{1.2}b, Ca\textsubscript{\textit{v}}\textsubscript{1.2}CM, or Ca\textsubscript{\textit{v}}\textsubscript{1.2}SM constructs (1.25 μg), together with β\textsubscript{2a} (1.25 μg) and α\textsubscript{2} (1.25 μg) using the calcium phosphate transfection method (8). The β\textsubscript{2a} and α\textsubscript{2} clones were provided by Dr. Terrance Snutch (University of British Columbia). After 48–72 h, I\textsubscript{Ba} was recorded at room temperature (23 °C) using the whole cell patch clamp technique. The external solution contained (in mM) 100 HEPES, 140 tetraethylammonium methanesulfonate, 5 BaCl\textsubscript{2}, or 1.8 CaCl\textsubscript{2} (pH was adjusted to 7.4 with CsOH and osmolarity to 290–310 with glucose). The internal solution (pipette solution) contained (in mM) 138 Cs-MeSO\textsubscript{4}, 5 CsCl, 0.5 EGTA, 10 HEPES, 1 MgCl\textsubscript{2}, 2 mg/ml Mg-ATP, pH 7.3 (adjusted with CsOH). Glucose was used to adjust the osmolarities of solutions to between 290 and 330 mOsm. The voltages are uncorrected for a 11-mV junction potential, and actual voltage can be obtained by subtracting 11 mV from the reported values.

Whole cell currents, obtained under voltage clamp with an Axopatch 200B amplifier (Axon Instruments), were filtered at 1–5 kHz and sampled at 5–50 kHz, and the series resistance was typically <5 megaohms after >70% compensation. The P/4 protocol was used to subtract online the leak and capacitive transients.

To determine the whole cell 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. In each cell, I\textsubscript{Ba} at all voltages was normalized to the peak current. 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. The steady-state inactivation curves were obtained from experiments by stepping from a holding poten-
of −90 mV to a 30-ms normalizing pulse to 10 mV followed by a family of 15-s long pulses from −120 to 20 mV. A 104-ms test pulse to +10 mV was recorded 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: 

\[ I_{\text{relative}} = I_{\text{min}} + \frac{I_{\text{max}} - I_{\text{min}}}{1 + \exp((V_{1/2} - V)/k)} \]

where \( I_{\text{relative}} \) is the normalized current; \( V_{1/2} \) is the potential for half-inactivation, and \( k \) is the slope factor. 

\( G - V \) curves were obtained from a tail activation protocol. The cells were activated by a 20-ms test pulse of variable voltage family from −60 to 120 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: 

\[ \frac{G}{G_{\text{max}}} = F_{\text{low}}/[1 + \exp((V_{1/2,\text{low}} - V)/k_{\text{low}})] + (1-F_{\text{low}})/[1 + \exp((V_{1/2,\text{high}} - V)/k_{\text{high}})] \]

where \( G \) is the tail current and \( G_{\text{max}} \) is the peak tail current, \( F_{\text{low}} \) is the fraction of low threshold component; \( V_{1/2,\text{low}} \) and \( k_{\text{low}} \) are the half-activation potentials and slope factors for the low and high threshold components and \( V_{1/2,\text{act}} \) was calculated when \( G = 0.5G_{\text{max}} \). \( \tau \) values for voltage dependence inactivation (in Ba²⁺ solution) were calculated from a single exponential equation: 

\[ I = I_{\text{min}} + I_{\text{max}}(\exp(-t/\tau)) \]

where \( I_{\text{min}} \) is the steady-state amplitude of the current, \( I_{\text{max}} \) is the initial current, and \( \tau \) is the time constant. Recovery from inactivation of \( I_{\text{Ba}} \) was investigated using a standard two-pulse protocol. Fractional recovery of peak \( I_{\text{Ba}} \) were plotted against \( \Delta T \). Inactivation time constants (\( \tau \)) were determined from the double exponential equation: 

\[ Y = Y_{\text{min}} + A_1 \times [1 - \exp(-t/\tau_1)] + A_2 \times [1 - \exp(-t/\tau_2)] \]

where \( Y \) is the fraction of recovery, \( A_1 \) and \( A_2 \) are the maximum values of the fast and slow component, and \( \tau_1 \) and \( \tau_2 \) are the time constants, respectively.

Nifedipine (Sigma) was dissolved in Me₂SO to make a 10 mM stock solution and stored at −20 °C. Nifedipine at 0.1–1000 nM concentrations was freshly prepared in bath solution from stock and then applied to the HEK 293 cells. All nifedipine solutions were stored in the dark. The cells were held at either −50 or −90 mV and a test pulse of 100 ms at 0 mV was applied with an interpulse interval of 30 s. Inhibition ratios were calculated by comparing the peak currents before and after drug treatment. All experiments involving nifedipine were conducted as much as possible in the absence of light.

Values are expressed as mean ± S.E. GraphPad Prism software was used for data plotting and statistical analysis. Statistical significance of differences between means was calculated with Student’s t test or one-way ANOVA. \( p < 0.05 \) is considered significant.

**RESULTS**

**Distribution of Exon 33 in Arteries and Hearts**—The Ca₉,1.2 calcium channels in cardiac and smooth muscles have been shown to differ at four alternatively spliced sites: exon 1/1a, exon 8/8a, exon 9*, and exon 31/32 (Fig. 1A) (19, 20). Exon 33 forms part of the IVS3–S4 linker region and in our previous study we showed that the exclusion of exon 33 could shift the activation potential of Ca₉,1.2 channels to more hyperpolarized direction (8, 24). In this paper, we investigated whether exon 33...
may demonstrate selective expression in smooth and cardiac muscles.

The expression of exon 33 was assessed by a RT-PCR colony screening method (8, 12, 25) that provides quantification of expression that may differ at levels not easily determined by real-time PCR. PCR amplicons spanning exons 30–35 appeared as single bands on 1.8% agarose gels for rat aorta and heart tissues (Fig. 1B). To differentiate +/Δexon 33 in the PCR products, which differed by 33 bp, the PCR products were cloned into pGEM-T Easy vector, transformed into DH10B bacteria, and PCR colony screened for size differences on 1.8% agarose gels. This method provided a means to quantify the level of expression of Ca\textsubscript{1.2} transcripts, isolated from aorta and heart, with the inclusion or exclusion of exon 33. Random clones were selected for DNA sequencing analysis to validate the correlation of PCR product size with the +/Δexon 33 genotype in the PCR amplicon. Around 60–120 colonies were screened in each rat heart or artery tissue. A representative agarose gel result of a colony PCR screen was shown in Fig. 1C. Screening of heart Ca\textsubscript{1.2} channels showed that the colonies expressed almost exclusively the inclusion of exon 33 (Fig. 1C, upper panel), whereas data from aorta tissue demonstrated that a number of Ca\textsubscript{1.2} channels excluded exon 33 (Fig. 1C, lower panel). DNA sequencing confirmed the PCR product of smaller size lacked exon 33, whereas the PCR product of larger size contained exon 33. To further delineate the expression of exon 33 in different regions of the heart and in aorta, we screened PCR products obtained from mRNAs isolated from 3 to 8 rats. Indeed, ~32% of the Ca\textsubscript{1.2} channels in aorta (n = 5 rats) excluded exon 33, whereas the distribution of Δ33 in heart (n = 3–8 rats) varies from 2% in the right ventricle to 5% in the left atrium (Fig. 1D). The total number of colonies screened varied from 190 to 585 for each heart sample or aorta. The expression of Δ33 is significantly different in all four regions of the heart when compared with aorta (p < 0.001, one-way ANOVA and Newman-Keuls test). However, there is no significant difference in the expression of Δ33 among the various regions of the heart (p = 0.87, one-way ANOVA). As the difference in expression of Δ33 was ~30%, it would be difficult to detect such minor changes consistently by the real-time PCR method. The colony screening method, however, is more sensitive to detect small variations in the expression of an alternatively spliced exon.

To test whether Δ33 expresses in smaller arteries, we harvested rat cerebral basilar arteries and mesentery arteries. A similar colony screening method was used to quantify Δ33 expression. Mesenteric arteries (n = 3 rats) displayed 17%, whereas basilar arteries (n = 3 rats) expressed 36% of Δ33Ca\textsubscript{1.2} channels. Both results were significantly different from the levels expressed in rat heart (Fig. 1E, p < 0.01, one-way ANOVA and Newman-Keuls test). We further investigated the expression of Δ33 in human arteries. Of 5 human radial arteries obtained from patients who underwent coronary artery bypass, the average expression of Δ33 was 24%, which is still significantly higher than in rat heart (Fig. 1E, p < 0.01, one-way ANOVA and Newman-Keuls test). These results indicate that Δ33 expression appears widely in various rat and human arteries.

The well known Ca\textsubscript{1.2}SM channels that contain exons 1/8/9*/32 could now be subdivided in a more defined manner into two subgroups: one group contained exon 33, whereas a minor group excluded exon 33 (Fig. 1A). In our previous work we have identified the smooth muscle Ca\textsubscript{1.2} channel, genotyped as 1/8/9*/32Δ33, named Ca\textsubscript{1.2}SM in this paper to represent ~10% of the total transcripts (26).

Ca\textsubscript{1.2}SM Channels Underlie a Hyperpolarized Window Current—To avoid Ca\textsuperscript{2+} dependent effects and also for a better comparison with previous reports on Ca\textsubscript{1.2}a and Ca\textsubscript{1.2}b channel properties, we first performed electrophysiological recordings using 5 mM Ba\textsuperscript{2+} as the charge carrier (18, 21–23). We found that the Ca\textsubscript{1.2}SM splice variant showed fast voltage-dependent inactivation during depolarization to V\textsubscript{max} than Ca\textsubscript{1.2}b channels (Fig. 2A) and Ba\textsuperscript{2+} influx was significantly reduced, during 0.9-s pulses to V\textsubscript{max} by about 15% (Fig. 2B). It is known that an increased Ba\textsuperscript{2+} concentration can accelerate current inactivation. We carefully investigated the voltage-dependent inactivation with matched peak current and found the faster inactivation is still pronounced in Ca\textsubscript{1.2}SM. We further calculated the time constant (τvalues) with a single exponential equation. The τ values from Ca\textsubscript{1.2}SM channels were significantly lower at 0 and 10 mV (Fig. 2C, P\textsubscript{0.6 mV} < 0.01 and P\textsubscript{1.0 mV} < 0.05, unpaired t test). It is very likely that the faster inactivation is due to the exclusion of exon 33 in the domain IV S3–S4 loop as the only difference between Ca\textsubscript{1.2}SM and Ca\textsubscript{1.2}b is the exclusion of exon 33 in Ca\textsubscript{1.2}SM. To further determine differences in biophysical properties of the two smooth muscle Ca\textsubscript{1.2} splice variants, we examined their activation and inactivation potentials by functionally expressing the splice variants in HEK 293 cells (Fig. 2D).

Our whole cell patch clamp electrophysiological recordings showed that Ca\textsubscript{1.2}SM channels activated and inactivated more negatively than the Ca\textsubscript{1.2}b channels (Fig. 2D). The V\textsubscript{1/2} of steady-state inactivation significantly shifted by -16.3 mV, whereas the V\textsubscript{1/2} of activation potential shifted by -11.3 mV (Fig. 2D and Table 1, p < 0.0001, unpaired t test). The slope factors between the two channels are similar in steady-state inactivation and the lower component of G–V curves (K\textsubscript{low}). The slope factor of Ca\textsubscript{1.2}SM on the higher component of G–V curves (K\textsubscript{high}) is larger than Ca\textsubscript{1.2}b channels with bigger standard error. The shift of both the activation and inactivation curves of Ca\textsubscript{1.2}SM channels generates a Ba\textsuperscript{2+} window current that peaked at -29.9 mV, compared with the peak at -20.8 mV for Ca\textsubscript{1.2}b channels. This 9 mV left-shifted window current of Ca\textsubscript{1.2}SM channel would permit the channels to maintain opening at hyperpolarized membrane potentials closer to resting potential than Ca\textsubscript{1.2}b channels. To test the activation and inactivation properties of both channels under physiological conditions, we performed experiments using 1.8 mM Ca\textsuperscript{2+} as the charge carrier. Although generally smaller currents were recorded in 1.8 mM Ca\textsuperscript{2+} bath solution, a similar left shift of window current was observed (Fig. 2E and Table 1). Both steady-state inactivation and activation potentials of Ca\textsubscript{1.2}SM channels are hyperpolarized shifted as compared with Ca\textsubscript{1.2}b channels (Ca\textsubscript{1.2}SM, V\textsubscript{0.5,inact} = -52.09 ± 0.6 mV, n = 12; Ca\textsubscript{1.2}b, V\textsubscript{0.5,inact} = -44.3 ± 0.35 mV, n = 8; p < 0.0001, unpaired t test; Ca\textsubscript{1.2}SM, V\textsubscript{0.5,act} = 2.19 ± 1.55 mV, n = 4; and Ca\textsubscript{1.2}b, V\textsubscript{0.5,act} =
inactivation (right) protocols and examples of their corresponding of current traces. The current traces were shown for determining activation property evoked at –60, –20, 20, 60, and 100 mV (left) and for inactivation property evoked at –100, –60, –20, and 20 mV (right). Plots of steady-state inactivation (f∞) and activation (d∞) curves of Ca_{1.2SM} (gray) and Ca_{1.2b} (black) channels derived from recordings performed in 5 mM Ba^{2+} (F), plots of steady-state inactivation (f∞) and activation (d∞) curves of Ca_{1.2SM} (gray) and Ca_{1.2b} (black) channels derived from recordings in bath solution containing 1.8 mM Ca^{2+} (E), plots of calculated calcium window currents of Ca_{1.2SM} (circles) and Ca_{1.2b} (filled circles) channels, as well as the combined window current (line). G, recovery from inactivation of I_{Ba} was investigated using a standard two-pulse protocol. Fractional recovery of peak I_{Ba} was plotted against ΔT.

Novel Smooth Muscle Ca_{1.2} Splice Variant

Exon 32 Expression Is Predominant in Cardiac Ca_{1.2} Channels—Exons 31 and 32 are mutually exclusive exons forming part of the IVS3 region (Fig. 1A). It was suggested that the cardiac isoform contained exon 31, whereas the smooth muscle isoform contained exon 32 (19, 20). Alternative splicing at this locus was demonstrated to influence DHP sensitivity slightly (31). However, we and others have reported that exon 32 is predominant in adult heart from various species (8, 10, 14, 32), and exon 31 was expressed at higher levels only in neonates (10). To further show that exon 32 is the predominant form in adult rat heart, we used restriction enzyme digestion by NsiI to identify the presence of exon 32 in the PCR products amplified from rat heart mRNA across exons 30–35. Digestion of the 382-bp product by NsiI that yielded two smaller fragments of 17.3 ± 1.38, n = 4; p = 0.0003, unpaired t test). To more carefully examine the Ca^{2+} window current, we calculated the window current by multiplying the activation conductance (d∞) curve by the inactivation curve (f∞) as reported previously by Chemin et al. (27). Current density from HEK 293-transfected cells was not included in the analysis. Calculated window currents from the 30% of Ca_{1.2SM} and 70% of Ca_{1.2b} channels were shown in Fig. 2F. The window current of Ca_{1.2SM} channels starts at –60.4 mV and peaked at –45.9 mV. The window current of Ca_{1.2b} channels starts at –55.5 mV and peaked at –41.07 mV. The combined window current of both channels represents around 1.5% of peak current. Taking into consideration that the 11 mV junction potential that was not corrected, the Ca_{1.2SM} channels (and some of Ca_{1.2b} channels) could be activated more readily close to the resting membrane potential of smooth muscles (between –75 to –60 mV in vitro, or –50 to –40 mV in vivo) (28) to maintain vasotone.

To further determine whether mechanistically the Ca_{1.2SM} channels may be more likely to open during high frequency stimulation, we determined the rate of I_{Ba} recovery of the channels from inactivation. A typical two-pulse protocol was used with a prepulse of 2-s duration chosen as in previous reports to better inactivate the channels (22, 29, 30). During 2-s prepulses of 0 mV from a holding potential of –90 mV, currents were reduced to around 30% (Ca_{1.2SM}, 28 ± 2%, n = 20; Ca_{1.2b}, 32 ± 2.3%, n = 13; p = 0.19, unpaired t test). A subsequent 40-mS test pulse was applied at variable time intervals (ΔT) after prepulses. After a second pulse to 0 mV, over 80% of all available channels recovered from the inactivated state within 4 s. We found that there was no difference in the recovery rate between the two smooth muscle Ca_{1.2} splice variants (Fig. 2G).
Novel Smooth Muscle Ca\textsubscript{1.2} Splice Variant

TABLE 1

|                      | Electrophysiological properties of Ca\textsubscript{1.2SM} and Ca\textsubscript{1.2b} channels | Steady-state inactivation |
|----------------------|-----------------------------------------------------------------------------------------------|--------------------------|
|                      | 5 mM Ba\textsuperscript{2+}                                                                  | 1.8 mM Ca\textsuperscript{2+} |
|                      | G–V                                                                                           | G–V                      |
|                      |                                                                                               |                          |
| \( V_{1/2} \)         | \( k_{\text{low}} \) \( k_{\text{high}} \)                                                    | \( V_{1/2} \)          |
| Ca\textsubscript{1.2SM} | \( -10.9 \pm 1.4 \) (8) 34.4 \pm 25.8                                                       | \( -50.8 \pm 1.2 \) (8) 15.8 \pm 6.4 |
| Ca\textsubscript{1.2b} | \( 0.4 \pm 1.2 \) (9) 17.3 \pm 4.8                                                          | \( -34.5 \pm 0.7 \) (7) 19 \pm 4.6 |

* \( p < 0.001 \) (unpaired \( t \) test).

314 and 68 bp would indicate the presence of exon 32, whereas the undigested 382 bp product would contain exon 31 instead (Fig. 3A). The results of this experiment clearly showed that heart Ca\textsubscript{1.2} channels expressed more abundantly exon 32 than exon 31 (Fig. 3B). We therefore constructed a new cardiac isoform of Ca\textsubscript{1.2} channel named Ca\textsubscript{1.2CM} (1a/8a/9a/32/33), which is a better representation of the adult cardiac isoform of Ca\textsubscript{1.2} channels than the Ca\textsubscript{1.2a} channels (Fig. 3C).

Steady-state Inactivation of Ca\textsubscript{1.2SM} Is More Hyperpolarized Shifted Than Ca\textsubscript{1.2CM} Channels—As the biophysical properties of Ca\textsubscript{1.2SM} and Ca\textsubscript{1.2CM} channels have not been evaluated together, we first compared their current-voltage (I–V) relationships by stepping to a family of test potentials from a holding potential of \(-90 \text{ mV}\). The current-voltage (I–V) curves showed no statistical difference between Ca\textsubscript{1.2CM} and Ca\textsubscript{1.2SM} channels (Fig. 4B and Table 2, Ca\textsubscript{1.2CM}, \( V_{0.5} = -27.49 \pm 1.32, n = 9 \) cells; Ca\textsubscript{1.2SM}, \( V_{0.5} = -24.68 \pm 1.09, n = 10 \) cells; \( p = 0.1164 \), unpaired \( t \) test). From the sample traces of I–V relations shown in Fig. 4A, we found that the voltage-dependent inactivation of Ca\textsubscript{1.2SM} was faster than Ca\textsubscript{1.2CM}. To characterize the voltage-dependent inactivation of both channels, we compared the time-dependent inactivation of \( V_{\text{max}} \) (Fig. 4C). The inactivation rate of Ca\textsubscript{1.2SM} is faster than Ca\textsubscript{1.2CM in} 0.3, 0.6, and 0.9 s, \( p_{0.3s} = 0.0007, p_{0.6s} = 0.00028, p_{0.9s} = 0.0074 \), unpaired Student’s \( t \) test (Fig. 4D). At 0 mV, the \( \tau \) values of Ca\textsubscript{1.2SM} channels are significantly smaller than Ca\textsubscript{1.2CM} channels (Fig. 4E) after fitting with a single exponential equation. To more accurately investigate activation potentials, we used the tail protocols and again obtained that there was no significant difference between the \( V_{1/2} \) act of both channels (Fig. 4F and Table 2, \( p = 0.4728 \), unpaired \( t \) test). Both I–V relationships and tail currents clearly showed the Ca\textsubscript{1.2SM} and Ca\textsubscript{1.2CM} shared similar activation properties. However, when we compared the voltage-dependent steady-state inactivation properties of both channels by providing a 15 s conditioning prepulse before stepping to test pulse at 10 mV, we detected an approximate \(-16 \text{ mV}\) hyperpolarized shift of \( V_{1/2} \) inact for Ca\textsubscript{1.2SM} over Ca\textsubscript{1.2CM} channels (Fig. 4F and Table 2, \( p < 0.0001 \), unpaired \( t \) test). Thus the Ca\textsubscript{1.2SM} channels own a smaller window current than Ca\textsubscript{1.2CM}.

Biophysical Property of Ca\textsubscript{1.2SM} Channels Linked to Enhanced Inhibition by Nifedipine—To further test our hypothesis that altered steady-state inactivation alone in a smooth muscle Ca\textsubscript{1.2} splice variant could also modulate sensitivity to DHP, nifedipine was used to block both smooth muscle splice variants, Ca\textsubscript{1.2SM} and Ca\textsubscript{1.2b}, and cardiac Ca\textsubscript{1.2CM} channels at various concentrations. As nifedipine blockade is state-dependent, we performed the whole cell electrophysiological recordings at two holding potentials (\( V_{h} = -90 \text{ or } -50 \text{ mV} \)) in bath solution containing 5 mM Ba\textsuperscript{2+}. The two holding potentials were chosen to better reflect the pharmacological responses of the channels held close to the reported resting potentials of smooth and cardiac muscles. We also performed experiments using more physiological conditions with 1.8 mM Ca\textsuperscript{2+} as the charge carrier. However, the current was too small when the cells were held at \(-50 \text{ mV} \). Experiments with 1.8 mM Ca\textsuperscript{2+} were therefore only conducted in the holding potential of \(-90 \text{ mV} \). Step depolarizations to 0 mV were applied every 30 s and Fig. 5A illustrates the diary plot of nifedipine inhibition. The cells were viable as after washing out the nifedipine, substantial recovery of \( I_{\text{Ba}} \) was observed (>85% recovery). Exemplary current traces from three channels, in the absence and presence of 10 nM nifedipine, are shown in Fig. 5B. All three channels were more sensitive to nifedipine under depolarized holding potentials. When the Ca\textsubscript{1.2} splice variants were characterized pharmacologically under the same holding potentials, we found that Ca\textsubscript{1.2SM} channels were the most potently inhibited by nifedipine, followed by Ca\textsubscript{1.2b} channels, whereas the Ca\textsubscript{1.2CM} channels exhibited the least inhibition. The dose-response curves fitted with the Hill equation clearly indicated that nifedipine inhibited Ca\textsubscript{1.2SM} channels between 5.4 and 6.4 times more potently than Ca\textsubscript{1.2b} and Ca\textsubscript{1.2CM} channels at the holding potential of \(-90 \text{ mV} \) and between 4.7 and 9.4 times at \(-50 \text{ mV} \), respec-
channel in that it lacks exon 33, but nonetheless, the $\text{Ca}_{\alpha,1.2}\text{SM}$ splice variant exhibited a hyperpolarized shift in steady-state inactivation property that was linked to enhanced sensitivity to nifedipine. These results clearly affirm the state-dependent block of nifedipine but more importantly, it provides another mechanism to explain the vascular selectivity of DHP inhibition of $\text{Ca}_{\alpha,1.2}$ channels in the cardiovascular system. Taken together, we have demonstrated the existence of a subpopulation of smooth muscle $\text{Ca}_{\alpha,1.2}\text{SM}$ splice variant that activated and inactivated close to resting potential and that were blocked more potently by nifedipine because of a hyperpolarized shift in steady-state inactivation potential.

**DISCUSSION**

In this report, we have characterized functionally a $\text{Ca}_{\alpha,1.2}$ splice variant that possesses electrophysiological and pharmacological properties that at least in part addressed two puzzles about smooth muscle L-type calcium channels. One is the presence of a subpopulation of DHP-sensitive $\text{Ca}_{\alpha,1.2}$ channels that open within a narrow window current that is close to the resting potential in smooth muscles. The second is the absence of $\text{Ca}_{\alpha,1.2}$ splice variants with biophysical properties that linked the inactivation state to block by DHP. We hypothesized that certain uncharacterized or novel smooth muscle $\text{Ca}_{\alpha,1.2}$ splice variants would underlie these two important vascular $\text{Ca}_{\alpha,1.2}$ channel properties. Unexpectedly, we found that a single $\text{Ca}_{\alpha,1.2}\text{SM}$ splice variant exhibited a hyperpolarized shift in $\text{Ca}^{2+}$ window current and was also more potently inhibited by nifedipine correlating with a left-shift in its steady-state inactivation potential. Given the characteristics of the $\text{Ca}_{\alpha,1.2}\text{SM}$ channels, it is not unreasonable to speculate that this splice variant could contribute to the basal level of $\text{Ca}^{2+}$ influx near resting potential and should therefore serve a critical purpose to underlie basal contractility in myocyte that is essential for vasotone and blood flow (3, 4).

The smooth muscle $\text{Ca}_{\alpha,1.2}\text{b}$ splice variant was shown to exhibit a window current that is similar to the cardiac $\text{Ca}_{\alpha,1.2}$ channel isoform (21). For the generation of vascular tone, a smooth muscle $\text{Ca}_{\alpha,1.2}$ channel splice variant that activates at more hyperpolarized potential is required as increase in smooth muscle tension is sensitive to changes in membrane potential, where the relationship between vascular tone and $V_{\text{m}}$ is steep in the potential range of $-65$ and $-40$ mV (28, 33). As such, the $\text{Ca}_{\alpha,1.2}\text{SM}$ splice variant will play a critical role in being responsive to activation close to the resting potential and upon further membrane depolarization, other splice variants can then be opened to contribute to the overall window current of smooth muscle $\text{Ca}_{\alpha,1.2}$ channels. It is of interest that the window currents of both $\text{Ca}_{\alpha,1.2}\text{SM}$ and $\text{Ca}_{\alpha,1.2}\text{b}$ channels are rather small and they peak around $-45.9$ and $-41.1$ mV. This $-5$ mV difference is important given that the open probability of the channels or the tension of the muscle changes e-fold for $6-8$ mV change over the physiological membrane potential negative to $-40$ mV (33). Therefore, by itself the $\text{Ca}_{\alpha,1.2}\text{SM}$ channels do not completely constitute the window current of native smooth muscles, but it should play an important role to contribute to the more hyperpolarized part of the overall win-
dow current and should be important to maintain steady-state resting vascular tone (3).

The molecular structure of Cav1.2 channels in cardiac and arterial smooth muscles are of particular interest as they are the targets of calcium channel blockers treating a variety of cardiovascular diseases. Previous reports identified two major isoforms of Cav1.2 channels generated through the differential utilization of alternatively spliced exons (19, 20). The Ca\(_{\text{a}}\).1.2a (cardiac form) and Ca\(_{\text{a}}\).1.2b (smooth muscle form) are different at 4 sites, namely exon 1/1a, exon 9*, exon 8/8a, and exon 31/32. Whereas the utilization of exon 1 or 1a is regulated by different promoters (34–36), many reports found that exon 32 instead of exon 31 is predominantly expressed in adult heart (8, 10, 14, 32). Thus a more accurate genotype of the predominant cardiac Ca\(_{\text{a}}\).1.2CM channel should contain alternatively spliced exons 1a/8a/9004/9005/32 instead of 1a/8a/9004/31. This isoform was found only in cardiac myocytes but not found in rat aorta (26). Nonetheless, from our previous report, the electrophysiological

TABLE 2
Electrophysiological properties of Ca\(_{\text{a}}\).1.2SM and Ca\(_{\text{a}}\).1.2CM channels in bath solution containing 5 mM Ba\(^{2+}\)

|             | IV G–V Steady-state inactivation |
|-------------|----------------------------------|
|             | \(V_{1/2}\) | \(k\) | \(V_{1/2}\) | \(k_{\text{low}}\) | \(k_{\text{high}}\) | \(V_{1/2}\) | \(k\) |
| Ca\(_{\text{a}}\).1.2CM | \(-27.5 \pm 1.3\) (9) | \(-7.5 \pm 0.7\) | \(-12.7 \pm 1.6\) (14) | \(9.5 \pm 0.6\) | \(18.39 \pm 21\) | \(-34.8 \pm 1.5\) (10) | \(-8.5 \pm 0.4\) |
| Ca\(_{\text{a}}\).1.2SM | \(-24.7 \pm 1.1\) (10) | \(-6.6 \pm 0.5\) | \(-10.9 \pm 1.4\) (8) | \(10.3 \pm 1.7\) | \(34.4 \pm 25.8\) | \(-50.8 \pm 1.2\) (8) | \(-9.8 \pm 0.4\) |

\(p < 0.0001\) (unpaired \(t\) test).

FIGURE 5. Voltage dependence of \(I_{\text{Ba}}\) inhibition by nifedipine. A, representative diary plots of peak current before, during, and after exposure to nifedipine. B, examples of current traces evoked from the holding potentials at either \(-90\) or \(-50\) mV for Ca\(_{\text{a}}\).1.2b, Ca\(_{\text{a}}\).1.2SM, or Ca\(_{\text{a}}\).1.2CM channels before (○) and after (□) the addition of 10 nM nifedipine. C and D, comparison of Ca\(_{\text{a}}\).1.2SM with Ca\(_{\text{a}}\).1.2b and Ca\(_{\text{a}}\).1.2CM channels blocked by nifedipine in 5 mM Ba\(^{2+}\) bath solution. Current amplitude in the presence of nifedipine was expressed as the percentage of control current amplitude without drug treatment. E, dose-response relationships of Ca\(_{\text{a}}\).1.2b, Ca\(_{\text{a}}\).1.2SM, and Ca\(_{\text{a}}\).1.2CM channels inhibited by nifedipine as measured in 1.8 mM Ca\(^{2+}\) external solution (\(V_{\text{h}}\) = \(-90\) mV).

TABLE 3
Inhibition of \(I_{\text{Ba}}\) and \(I_{\text{Ca}}\) by nifedipine

The IC\(_{50}\) values were calculated from the dose-response curves fitted with Hill equation.

|             | \(-90\) mV | \(-50\) mV | \(-90\) mV |
|-------------|------------|------------|------------|
|             | Slope      | Slope      | Slope      |
| Ca\(_{\text{a}}\).1.2SM | \(13.5 \pm 1.2\) (23) | \(-0.53 \pm 0.07\) | \(2.8 \pm 1.4\) (16) | \(-0.64 \pm 0.05\) |
| Ca\(_{\text{a}}\).1.2CM | \(85.9 \pm 1.1\) (23) | \(-0.69 \pm 0.03\) | \(26.7 \pm 1.2\) (30) | \(-0.5 \pm 0.02\) |

|             | \(-90\) mV | \(-50\) mV | \(-90\) mV |
|-------------|------------|------------|------------|
|             | Slope      | Slope      | Slope      |
| Ca\(_{\text{a}}\).1.2SM | \(13.3 \pm 1.1\) (27) | \(-0.7 \pm 0.13\) | \(13.4 \pm 1.1\) (23) | \(-0.78 \pm 0.03\) |
| Ca\(_{\text{a}}\).1.2b | \(100.4 \pm 4.6\) (15) | \(-0.55 \pm 0.37\) | \(25.7 \pm 1.3\) (28) | \(-0.9 \pm 0.19\) |

\(p < 0.001\), compared to Ca\(_{\text{a}}\).1.2SM channels (one-way ANOVA and Newman-Keuls test).
properties of exon 31 or exon 32 containing Ca\textsubscript{1.2} channels were similar. Whereas we have detected use of the alternate donor site for exon 32, we have not been able to discover similar alternative splicing at the donor splice junction of exon 31 (8). Whether this is the only functional difference arising from alternative splicing at the exon 31/32 locus is still unknown.

In this report, we found another alternatively spliced exon 33, which forms part of the extracellular IVS3–4 linker, to possess different expression patterns in heart and arteries. Approximately 30\% of Ca\textsubscript{1.2} channels within rat aorta lacked exon 33, whereas over 95\% of rat heart Ca\textsubscript{1.2} calcium channels do express exon 33. Thus the arterial smooth muscles contained at least two isoforms of Ca\textsubscript{1.2} calcium channels: one is the traditional Ca\textsubscript{1.2}b, and the other is Ca\textsubscript{1.2}SM. Importantly, we found that beside aorta, other arteries with smaller lumen, like rat mesenteric and cerebral arteries and human radial arteries, contained variable but significant amounts of Ca\textsubscript{1.2} channels lacking exon 33. The relevance of Ca\textsubscript{1.2}SM channels to smooth muscle function in arteries is therefore not restricted to the aorta but will be of more general significance in the vascular system. Different expression levels of exon 33 have been detected by RT-PCR by Feron et al. (37) in rat heart and rat smooth muscle cell line A7r5, where 7\% of Ca\textsubscript{1.2} channels in rat heart and 29\% in A7r5 lacked exon 33. This expression pattern is similar to what we found in rat heart and aorta. In our previous report (26), we have shown that Ca\textsubscript{1.2}SM channels represented ~10\% of the total full-length Ca\textsubscript{1.2} channel transcripts detected in aortic smooth muscles, whereas no Ca\textsubscript{1.2}CM splice variants were detected. To determine how such a small subpopulation of Ca\textsubscript{1.2}SM channels will contribute sufficiently to vasotone will require more work in the future. It is, however, noteworthy that in patients suffering from Timothy’s syndrome, a mutation found in exon 8a of the Ca\textsubscript{1.2} gene, which accounted for 11.5\% of total Ca\textsubscript{1.2} channels, die from arrhythmias and sudden cardiac death (12). One possible genetic approach is to develop a mouse that is deleted of exon 33 in the genome and the prediction is the generation of a larger number of Ca\textsubscript{1.2}SM channels and therefore the development of increased vasotone and blood pressure.

Previous studies on Ca\textsubscript{1.2}a and Ca\textsubscript{1.2}b found no difference in the channel gating properties such as activation and steady-state inactivation potentials (18, 21, 22). In contrast, Ca\textsubscript{1.2}SM channels displayed a prominent hyperpolarized shift in steady-state inactivation potentials compared with Ca\textsubscript{1.2}CM and Ca\textsubscript{1.2}b channels. Furthermore, pharmacological study of the three channels indicated that nifedipine blocked them in a voltage-dependent manner. All channels were more sensitive to nifedipine when the holding potential was at −50 mV compared with −90 mV. It is reasonable as DHPs tend to bind inactivated channels rather than closed or open channels and at −50 mV holding potential, more channels are in the inactivated state. When we compared Ca\textsubscript{1.2}SM with Ca\textsubscript{1.2}CM channels, we found that Ca\textsubscript{1.2}SM channels are much more sensitive to nifedipine blockade. Even at −90 mV HP, the IC\textsubscript{50} of Ca\textsubscript{1.2}SM is less than that of Ca\textsubscript{1.2}CM at −50 mV HP. Hence, from previous studies, the first mechanism to explain the ratio of vascular:cardiac DHP sensitivity was the more depolarized resting potentials of smooth muscles (17, 38). The first mechanism exploits the more depolarized membrane potential in smooth muscle membrane (17). The Ca\textsubscript{1.2}b channels would therefore be more likely to be shifted to the inactivated states after activation. As DHPs bind preferentially to the inactivated Ca\textsubscript{1.2}b channels (39–41), DHPs thus showed a high vascular selection in native smooth muscles (5, 42, 43). The second mechanism operates via the alternative splicing of the domain IS6 segment, which is encoded by mutually exclusive exons 8/8a that influenced the inhibitory effects of DHPs on the channels in a voltage-dependent manner. As such the different localizations and structures of cardiac and smooth muscle Ca\textsubscript{1.2} channels determine their DHP sensitivities (18, 21, 23, 31). However, with the different inactivation properties of Ca\textsubscript{1.2}SM channels, we now provide a third mechanism to explain tissue selectivity for DHP inhibition. Here, for the third mechanism, Ca\textsubscript{1.2}SM channel demonstrated a large hyperpolarized shift in voltage-dependent inactivation and this gating property confers on the smooth muscle channel variant enhanced sensitivity to nifedipine, thus linking biophysical property to potency of nifedipine block.

To test the precise role of the Ca\textsubscript{1.2}SM channel splice variant, specific knock-down of these variants for biological assessments of vascular tone or blood pressure are required. Nonetheless, in the management of hypertension with DHP, it is conceivable that Ca\textsubscript{1.2}SM channels can be targeted first to block their activities resulting in decreased vessel tone, followed by the blockade of other smooth muscle Ca\textsubscript{1.2} channel splice variants that activate at more depolarized potentials. A selective inhibition of the Ca\textsubscript{1.2}SM channels in native smooth muscles that is efficacious in the management of hypertension or hypertension-related vascular disorders will certainly be of clinical relevance and interest.

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