Atherosclerosis-related molecular alteration of the human $\text{Ca}_v1.2$ calcium channel α_{1C} subunit

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Atherosclerosis is an inflammatory process characterized by proliferation and dedifferentiation of vascular smooth muscle cells (VSMC). $\text{Ca}_v1.2$ calcium channels may have a role in atherosclerosis because they are essential for Ca^{2+} -signal transduction in VSMC. The pore-forming $\text{Ca}_v1.2\alpha_1$ subunit of the channel is subject to alternative splicing. Here, we investigated whether the $\text{Ca}_v1.2\alpha_1$ splice variants are affected by atherosclerosis. VSMC were isolated by laser-capture microdissection from frozen sections of adjacent regions of arteries affected and not affected by atherosclerosis. In VSMC from nonatherosclerotic regions, RT-PCR analysis revealed an extended repertoire of $\text{Ca}_v1.2\alpha_1$ transcripts characterized by the presence of exons 21 and 41A. In VSMC affected by atherosclerosis, expression of the $\text{Ca}_v1.2\alpha_1$ transcript was reduced and the $\text{Ca}_v1.2\alpha_1$ splice variants were replaced with the unique exon-22 isoform lacking exon 41A. Molecular remodeling of the $\text{Ca}_v1.2\alpha_1$ subunits associated with atherosclerosis caused changes in electrophysiological properties of the channels, including the kinetics and voltage-dependence of inactivation, recovery from inactivation, and rundown of the Ca^{2+} current. Consistent with the pathophysiological state of VSMC in atherosclerosis, cell culture data pointed to a potentially important association of the exon-22 isoform of $\text{Ca}_v1.2\alpha_1$ with proliferation of VSMC. Our findings are consistent with a hypothesis that localized changes in cytokine expression generated by inflammation in atherosclerosis affect alternative splicing of the $\text{Ca}_v1.2\alpha_1$ gene in the human artery that causes molecular and electrophysiological remodeling of $\text{Ca}_v1.2$ calcium channels and possibly affects VSMC proliferation.

alternative splicing | cell proliferation | vascular smooth muscle cells

Atherosclerosis is considered an inflammatory process that causes endothelial perturbation; local release of cytokines; and dedifferentiation, proliferation, and migration of vascular smooth muscle cells (VSMC) (1). Arterial VSMC constitute the media of the artery and play a crucial role in its elasticity and contractility. Contraction of VSMC is triggered by Ca^{2+} current through the voltage-gated $\text{Ca}_v1.2$ channels that are targets of Ca^{2+} -channel-blocking drugs (2, 3). The vasodilating effect of these drugs is associated with high affinity binding to the pore-forming α_{1C} subunit of the $\text{Ca}_v1.2$ channel (4) that in the case of dihydropyridines depends on membrane potential (5–8). The expression of $\text{Ca}_v1.2$ changes during cellular differentiation and proliferation, and is strongly affected by hormones and cytokines (9–11). The $\text{Ca}_v1.2\alpha_1$ subunit gene is subject to complex alternative splicing (12–20) that may change both pharmacological (21–23) and physiological properties (14, 21, 24, 25) of the channel.

Although splice variations in segments of the vascular $\text{Ca}_v1.2\alpha_1$ transcripts have been recently established (13, 14, 20), the relationship between distinct $\text{Ca}_v1.2\alpha_1$ splice isoforms and vascular disease has not yet been investigated. Our study of $\text{Ca}_v1.2\alpha_1$ splice variants in VSMC is the first attempt to identify changes in the human Ca^{2+} channel associated with atherosclerosis. Our findings revealed an extended repertoire of the exon-21 $\text{Ca}_v1.2\alpha_1$ splice isoforms in nonatherosclerotic VSMC and established a potentially important switch to a unique exon-22 isoform as a molecular signature of the

electrophysiologically remodeled proliferating pathophysiological state of VSMC in atherosclerosis.

Results

Reduced Expression of $\text{Ca}_v1.2\alpha_1$ in Atherosclerotic Regions of Human Artery. The Ca^{2+} current through the L-type $\text{Ca}_v1.2$ channels triggers contraction of VSMC (26). We sought to characterize the $\text{Ca}_v1.2\alpha_1$ transcripts in VSMC affected (VSMC^D, diseased) and not affected (VSMC^N, nondiseased) by atherosclerosis. VSMC were identified and isolated from the tissue by laser-capture microdissection (LCM). Arterial tissue obtained during vascular surgery procedures (three femoral and three carotid arteries; see Table 2, which is published as supporting information on the PNAS web site) was prepared in 5- to 7- μm , frozen sections from the regions of atherosclerotic plaques and adjacent control areas that had no evidence of atherosclerosis. Fig. 1 shows representative immunohistochemical patterns of the tissue sections used for LCM. VSMC were identified (Fig. 1A and F) in frozen sections by immunostaining with antibody against SM α -actin (27), used as a marker for VSMC. The SM α -actin staining correlated with immunostaining by anti- α_{1C} antibody in serial sections (Fig. 1B and G). Consistent with dedifferentiation of VSMC^D (28, 29) immunostaining against both the SM α -actin and α_{1C} was visually reduced in atherosclerotic regions (Fig. 1G). To quantify the $\text{Ca}_v1.2$ transcript, we identified 200–300 VSMC in atherosclerotic and unaffected regions of the artery by rapid SM α -actin immunostaining and then isolated them by LCM. RNA extracted from the cells was then analyzed by quantitative real-time PCR with SYBR green. By studying six different preparations, we determined that the relative α_{1C} mRNA level in VSMC (normalized to 18S RNA) was reduced 3.7 ± 0.9 fold (mean $\pm$ SEM) in the atherosclerotic region as compared with the adjacent nondiseased tissue ($P < 0.02$, paired t test). This result confirms that atherosclerosis causes reduction in expression of the $\text{Ca}_v1.2$ channels in VSMC.

Reduced $\text{Ca}_v1.2$ expression was previously observed in human fibroblasts in response to mitogenic stimulation (9). The reduction in expression of the vascular $\text{Ca}_v1.2$ channels may also be due to mitogenic factors of local inflammation in the atherosclerotic plaque region that induce migration and dedifferentiation of VSMC (1, 28, 29). The nonatherosclerotic artery contains very few proliferating cells (Fig. 1C) and is characterized by very limited presence of cytokines such as PDGF-BB (Fig. 1D) and its receptor PDGFR- β (Fig. 1E). By contrast, in atherosclerotic regions there

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Abbreviations: VSMC, arterial vascular smooth muscle cells; VSMC^D, diseased (atherosclerotic) VSMC; VSMC^N, nondiseased (nonatherosclerotic) VSMC; LCM, laser-capture microdissection; mRNA^{Card}, human cardiac mRNA.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. AY830711–AY830713, Z34811, and Z34812).

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Fig. 1. Representative immunohistochemical patterns of the vascular preparations used for LCM of VSMC and isolation of RNA from atherosclerotic (D) and adjacent nonatherosclerotic (N) regions of artery. Shown are photomicrographs of immunohistochemical staining of VSMC in serial sections of the same biopsy of arteries with antibodies against smooth muscle (SM) α -actin (A and F) (for the individual patient data, see Fig. 6), $\text{Ca}_v1.2\alpha1$ (B and G), ubiquitous human nuclear protein Ki-67 (C and H), PDGF-BB (D and I), and PDGF- β receptor (E and J). (Scale bar: 50 μm .)

were much larger numbers of proliferating cells (Fig. 1H) that occur on conjunction with elevated PDGF-BB (Fig. 1I) and PDGFR- β (Fig. 1J). Consistent with effects of atherogenesis (29), the shape and arrangement of VSMC were changed (compare Fig. 1A and F). Taken together, these data characterize quantitative differences between the unaffected and diseased regions of the arterial biopsies used in this work.

Molecular Remodeling of $\text{Ca}_v1.2\alpha1$ Associated with Atherosclerosis.

To determine whether the altered expression of $\text{Ca}_v1.2\alpha1$ in VSMC in atherosclerosis involves specific splice isoform(s), identity of the alternative exons of the $\text{Ca}_v1.2\alpha1$ subunit (Fig. 2A) was determined by RT-PCR. Arterial preparations from the six patients (Table 2) were investigated to substantiate the statistical significance of the results. Each of them (see examples in Fig. 7, which is published as supporting information on the PNAS web site) showed the same pattern of alternative splicing of $\text{Ca}_v1.2\alpha1$ in VSMC^N and its change in VSMC^D is summarized in Fig. 2B–G. With a primer complementary to conserved exon 3, sense primers P283 and 22 $\gg$ 38 (see Fig. 2 legend) generate PCR products of 541 and 419 bp only when exon 1a and exon 1, respectively, are present. By using this assay, exon 1a was identified in the control human cardiac mRNA ($\text{mRNA}^{\text{Card}}$) (Fig. 2B, lane 1), but it was absent from the VSMC^N transcripts (Fig. 2B, lanes 3 and 5). However, exon 1 was found in both $\text{mRNA}^{\text{Card}}$ (lane 2) and RNA isolated from VSMC^N (lane 4) and VSMC^D (Fig. 2B, lane 6). Thus, the N terminus of the human vascular $\text{Ca}_v1.2\alpha1$ is encoded by exon 1 and does not change in atherosclerosis.

To discriminate between exons 8A and 8, RT-PCR products obtained with indicated primers (Fig. 2C) were digested with BamHI. Exon 8A has a unique BamHI restriction site that yields the 478- and 207-bp fragments from the 685-bp RT-PCR product. Both the exon 8 and exon 8A species of the $\text{Ca}_v1.2\alpha1$ transcripts were positively identified in $\text{mRNA}^{\text{Card}}$ (Fig. 2C, lane C), whereas only the BamHI-resistant exon 8-isoform of the α_{1C} transcripts was observed in VSMC (Fig. 2C, lanes N and D). Previously it was reported that vascular $\text{Ca}_v1.2\alpha1$ incorporates the 75-bp combinatorial exon 9a between exons 9 and 10 (13, 14, 30, 31). We identified exon 9a in at least three VSMC^N $\text{Ca}_v1.2\alpha1$ subunit transcripts (Fig. 8, which is published as supporting information on the PNAS web site) corresponding to the 1,170- and 1,095-bp PCR products (Fig. 2D, lane N). However, only the 1,095-bp DNA was amplified from VSMC^D , indicating that exon 9a was absent from the VSMC^D $\text{Ca}_v1.2\alpha1$ (Fig. 2D, lane D).

The identity of alternative exons 21/22 was established by analytical AvrII digestion (Fig. 2E). Exon 22 has an AvrII restriction site that yields the 459- and 142-bp fragments from the 601-bp PCR product. By using AvrII digestion, the exon 22-isoform of α_{1C} was positively identified only in VSMC^D (Fig. 2E, lane D right). In contrast, VSMC^N (Fig. 2E, lane N) predominantly express the exon 21-isoform of the $\text{Ca}_v1.2\alpha1$ transcript that is resistant to AvrII.

Direct DNA sequencing of the crude PCR amplification products independently confirmed that result (Fig. 9, which is published as supporting information on the PNAS web site). Indeed, no distortion of the nucleotide peaks in the region of exon 21/22 was seen when compared with the exon-20 invariant region, suggesting that a switch to the exon-22 isoform of the vascular $\text{Ca}_v1.2\alpha1$ subunit was almost complete in atherosclerosis.

RT-PCR applied to the region of exons 30–34 generated several amplification products identified by analytical restriction digestion and direct sequencing (Fig. 2F). Analytical digestion by NsiI (Fig. 2F, lanes 3 and 4) and PvuII (Fig. 2F, lanes 5 and 6) confirmed the structural assessment summarized in Fig. 3 (boxed sequences). Only the exon-32 isoform of $\text{Ca}_v1.2\alpha1$, digested by NsiI into the 353- and 223-bp fragments, was identified in VSMC^D ($\alpha_{1C,77}$; Fig. 3). In contrast, four exon-32 isoforms of $\text{Ca}_v1.2\alpha1$ ($\alpha_{1C,71}$, $\alpha_{1C,73}$, $\alpha_{1C,125}$, and $\alpha_{1C,126}$), and the exon-31 isoform resistant to NsiI ($\alpha_{1C,127}$) were identified in VSMC^N . Analytical digestion by PvuII (Fig. 2F, lane 5) revealed heterogeneity of $\text{Ca}_v1.2\alpha1$ in VSMC^N due to exon 33. Exon 33 was found to be missing in two of five $\text{Ca}_v1.2\alpha1$ transcripts in VSMC^N ($\alpha_{1C,73}$ and $\alpha_{1C,125}$ in Fig. 2F). Additional heterogeneity in this regions of the structure was due to a 6-nt deletion (14, 20) in exon 32 ($\alpha_{1C,125}$) and upstream extension of exon 34 ($\alpha_{1C,126}$) in vascular $\text{Ca}_v1.2\alpha1$ splice isoforms.

Finally, RT-PCR from the region of exons 40–46 revealed single 858- and 801-bp products of amplification in VSMC^N and VSMC^D RNA, respectively (Fig. 2G). DNA sequencing showed that the difference is due to the 57-bp combinatorial exon 41A that was absent from the VSMC^D RNA. However, alternative exons 40B, 43A, and 45 (18) and the cardiac 213-bp exon 44A (16) are absent from the vascular $\text{Ca}_v1.2\alpha1$ transcripts. No additional heterogeneity was observed from the distal 3'-terminal region (data not shown).

Fig. 3 summarizes the results of the assessment of alternative splicing of the $\text{Ca}_v1.2\alpha1$ transcripts. In VSMC^N , we identified an extended repertoire of $\text{Ca}_v1.2\alpha1$ isoforms, all characterized by the presence of exons 21 and 41A. This selective heterogeneity of the $\text{Ca}_v1.2\alpha1$ subunits was replaced in VSMC^D by the single exon-22 variant lacking exon 41A.

Electrophysiological Remodeling of $\text{Ca}_v1.2$ Calcium Channels in Atherosclerosis. To determine whether molecular remodeling of $\text{Ca}_v1.2\alpha1$ in atherosclerosis causes changes in electrophysiological properties of the VSMC $\text{Ca}_v1.2$ calcium channel, $\alpha_{1C,77}$ of VSMC^D was compared with its splice variants in VSMC^N . Our goal here was to determine relative effects of the identified splicing variations on kinetics of inactivation, the $I-V$ relationship, steady-state inactivation, recovery from inactivation and run-down of the channels with Ba^{2+} and Ca^{2+} as charge carriers. Because we have not established yet whether splice variation of β subunits is affected by atherosclerosis, for comparative analytical measurements all channels were expressed with the β_{1a} accessory subunit that provides for intermediate kinetics of inactivation (24). In a number of previous

Fig. 2. Identification of the *Ca_v1.2 α 1* splice variants. (A) Hypothetical transmembrane topology of *Ca_v1.2 α 1* (17, 18). Outlined are four internal repeats, I–IV, each composed of six transmembrane segments, S1–S6. Protein segments encoded by numbered exons are marked by bold lines. Arrows point to alternative exons (8/8A, 21/22, and 31/32) that are subject to mutually exclusive splicing. Constitutively spliced exons 1/1A, 9A, 33, 34A, 41A, and 45 are shown by white boxes. (B–G) Identification of alternative exons of the *Ca_v1.2 α 1* transcript. (B) Exon 1a (lanes 1, 3, and 5) and exon 1 (lanes 2, 4, and 6). P283 is the exon 1a-specific primer 5'-tggatccgcaATGCTTCGAGCCTTGTCTAGC-3'. (C) Exons 8A and 8. (D) Exon 9a and 9. (E) Exons 21 and 22. (F) Exons 31–34. Shown are RT-PCR products (lanes 1 and 2) and their analytical digestion with NsiI (lanes 3 and 4) and PvuII (lanes 5 and 6). Splice variants identified by numbers on the left side of the gel photograph correspond to $\alpha_{1C,127}$ (1), $\alpha_{1C,73}$ (2), $\alpha_{1C,125}$ (3), $\alpha_{1C,126}$ (4), $\alpha_{1C,71}$ (5) and $\alpha_{1C,77}$ (6) (for details, see Fig. 10). (G) Differential utilization of exon 41A and lack of alternative exons 40B, 43A, 44A, and 45. Schematic diagrams illustrate the arrangement of alternative exons (black boxes) in RT-PCR products amplified from human mRNA^{Card} (lanes C) and RNA extracted from VSMC^N (lanes N) and VSMC^D (lanes D). Exons (boxes) are numbered as in A. The missing exons are shown as gray boxes. Numerals separated by $\gg$ or $\ll$ indicate the sense and antisense amplification primers, respectively, defined by nucleotide positions relative to the ORF of pHLCC71. To the right of schematics are RT-PCR products identified on agarose gels and their size in base pairs (arrows).

studies (14, 30, 31), effects of exon 9a incorporation on the channel properties have been characterized as marginal. Therefore, to simplify the interpretation of the results, we compared $\alpha_{1C,77}$ with the exon 9a-deficient $\text{Ca}_v1.2\alpha 1$ splice variants $\alpha_{1C,127}$, $\alpha_{1C,73}$, $\alpha_{1C,125}$, $\alpha_{1C,126}$, and $\alpha_{1C,71}$ detected in VSMC^N (Fig. 3). Major results are summarized in Table 1; see also Figs. 10 and 11, which are published

as supporting information on the PNAS web site. All tested channels generated a μA maximum I_{Ba} and I_{Ca} that was sufficiently large to disregard contribution from endogenous channels (50–80 nA). Analysis of I_{Ba} revealed that kinetics of voltage-dependent inactivation is changed only slightly between the $\alpha_{1\text{C},77}$ channel and splice variants of VSMC^N. The I - V relationships for all isoforms (Table 3, which is published as supporting information on the PNAS web site) show peak I_{Ba} at +10 mV. However, voltage-dependence of activation and inactivation are both significantly ($P < 0.05$) different in $\alpha_{1\text{C},77}$, causing a notable change in the slope of the activation curve and a shift of the steady-state inactivation curve to more positive voltages. Confirming previous observations (24), these data indicate that sensitivity of the $\alpha_{1\text{C},77}$ channel to voltage gating is altered as compared with the tested VSMC^N channels.

Replacement of Ca^{2+} for Ba^{2+} as the charge carrier evoked Ca^{2+} -dependent inactivation that accelerated the I_{Ca} decay in all tested channels (Table 1). Unlike I_{Ba} , Ca^{2+} currents reached maximum at approximately +20 mV and exhibited U-shaped dependence of τ_i on membrane potential (Fig. 11B) characteristic for Ca^{2+} -dependent inactivation (24, 32) that becomes faster with larger current. Although with the β_{1a} subunit (40 mM Ca^{2+}) kinetics of inactivation of I_{Ca} varies between the different channel isoforms expressed in VSMC^N and compared with the $\alpha_{1C,77}$ channel, no significant difference was observed between the $\alpha_{1C,77}$ and $\alpha_{1C,127}$ channels with the primary cardiac β_{2a} subunit (2.5 mM Ca^{2+} ; Fig. 4A). Another interesting finding is that two of the tested VSMC^N channels, $\alpha_{1C,125}$ and $\alpha_{1C,127}$ with the β_{1a} subunit, showed a significant ($P < 0.05$) sustained component of I_{Ca} that comprised 5.4% and 4.7%, respectively, of the total I_{Ca} by the end of a 1-s test pulse (Table 1). Contrary to the Ba^{2+} current data, significant changes of the voltage-dependence of the I_{Ca} activation and inactivation of the $\alpha_{1C,77}$ channel were found only with $\alpha_{1C,127}$ and observed with both β_{1a} and β_{2a} subunits. With 2.5 mM Ca^{2+} in the bath solution, this difference was even greater, and the channel assembled of the $\alpha_{1C,77}$ and β_{2a} subunits showed a 15-mV shift of the I - V relation (Fig. 4B) and the activation curve (Fig. 4C) by ≈ 15 mV in the hyperpolarizing direction.

Recovery of the current from inactivation was measured with +10 mV (Ba^{2+}) or +20 mV (Ca^{2+}) prepulses of a 1-s duration followed by increasing time intervals (25 ms to 1 s) at -90 mV before 0.25-s test pulses to +10 mV (Ba^{2+}) or +20 mV (Ca^{2+}) were applied. The ratio of the maximum current, evoked in response to a prepulse, to that of the test pulse was calculated as a fraction of the current. All tested channels showed slower recovery from inactivation as compared with $\text{Ca}_v1.2\alpha1$ mutants deprived of Ca^{2+} -dependent inactivation (24). The Ca^{2+} -conducting $\alpha_{1C,77}$ channel recovered from inactivation significantly ($P < 0.05$) faster than $\alpha_{1C,127}$ (Fig. 4E), but no significant difference was observed between the tested Ba^{2+} -conducting channels (Fig. 10E). Finally, all tested channels showed typical run-down (10% of $I_{\max}$ in 4 min) except for $\alpha_{1C,127}$ [Figs. 4F (Ca^{2+}) and 10F (Ba^{2+})]. Taken together, these data revealed that a number of important electrophysiological properties of the $\alpha_{1C,77}$ channel in VSMC^D are changed from those of the VSMC^N channel isoforms.

Possible Association of the Exon-22 Isoform of Ca_v1.2 α 1 and Proliferation of VSMC. It is known that in response to locally elaborated cytokines, VSMC^D assume a proliferative phenotype and migrate in the atherosclerotic plaque area. To determine whether VSMC proliferation may be associated with reduced expression and the isoform switch of the Ca_v1.2 α 1 subunit, we examined effects of serum deprivation of human coronary artery SM cells in culture on alternative splicing of exons 21/22 and level of α_{1C} transcripts. Identity of the cells was established by the manufacturer (Clonetics, San Diego, CA) by positive staining for smooth muscle α -actin and negative staining for von Willebrand's factor VII. To halt cell proliferation, VSMC were grown to a confluent monolayer, and then the confluent cell culture was subjected to serum deprivation

	exon 31/32	exon 33	exon 34a
$\alpha_{1C,127}$	GYFSDPWNVDFLIVIGSIIDVILSETN	PAEHTQCSPSPM	-----
$\alpha_{1C,73}$	HYFCDAWNTFDALIVVGSIVDIAITEVN	-----	-----
$\alpha_{1C,125}$	HYFCDAWNTFDALIVVGSIVDIAITE--	-----	-----
$\alpha_{1C,126}$	HYFCDAWNTFDALIVVGSIVDIAITEVN	PAEHTQCSPSPM	GPSCSHPLAVLTAPPVADGFQ
$\alpha_{1C,71}$	HYFCDAWNTFDALIVVGSIVDIAITEVN	PAEHTQCSPSPM	-----
$\alpha_{1C,77}$	HYFCDAWNTFDALIVVGSIVDIAITEVN	PAEHTQCSPSPM	-----

Fig. 3. Distribution of alternative exons in transcripts of the $\text{Ca}_v1.2\alpha1$ splice isoforms identified in VSMC^N and VSMC^D. Amino acid sequences encoded in alternative exons 31–34 are shown (boxes) beneath the chart. The α_{1C} subunit isoforms indicated on the left correspond to electrophysiologically characterized variants lacking exon 9a.

for 4 days. Under these conditions, DNA biosynthesis (assessed as [³H]thymidine incorporation) was decreased by 92%, the level of the $\text{Ca}_v1.2\alpha1$ transcript increased 2.8 ± 0.12 fold ($n = 3$, 27- and 32-year-old male donors), and the AvrII-sensitive exon-22 isoform of the $\text{Ca}_v1.2\alpha1$ transcript was not detected (Fig. 5, lane SF). However, when proliferation of nonconfluent VSMC was stimulated by the presence of 5% serum, DNA biosynthesis was restored and the presence of the exon-22 isoform of $\text{Ca}_v1.2\alpha1$ was detected (Fig. 5, lane S). Although the isoform switch was not complete as in atherosclerosis, the cell culture results suggest that in a very different experimental system there is an association between proliferation of VSMC, decreased expression of the $\text{Ca}_v1.2\alpha1$ transcript and appearance of the exon-22 isoform.

Discussion

This comprehensive study characterizes naturally occurring $\text{Ca}_v1.2\alpha1$ splice variants in human arterial VSMC and their remodeling in atherosclerosis. The conclusions are that VSMC in the regions of atherosclerotic plaques as compared with cells from control nonaffected portions of the same artery have reduced

expression of the $\text{Ca}_v1.2\alpha1$ transcript. This is accompanied by replacement of multiple exon-21 isoforms of the $\text{Ca}_v1.2\alpha1$ subunit with the single exon-22 isoform. That isoform exhibits altered electrophysiological properties and shows a possible association with VSMC proliferation.

Interestingly, two of the five investigated $\text{Ca}_v1.2\alpha1$ isoforms of VSMC^N exhibited important functional differences from other ones: the $\alpha_{1C,125}$ channel showed a significant residual component of I_{Ca} by the end of the 1-s depolarization (Table 1), whereas $\alpha_{1C,127}$ exhibited slower recovery of I_{Ca} from inactivation and lack of run-down of both I_{Ba} and I_{Ca} (Figs. 10 and 11). These findings raise an interesting possibility that some $\text{Ca}_v1.2$ channel isoforms are less sensitive to Ca^{2+} -induced inactivation that controls both the slow inactivation (33) and run-down of the channel (34). Because $\alpha_{1C,125}$ and $\alpha_{1C,127}$ are able to maintain a more sustained Ca^{2+} flux, they may have a specific role in VSMC functions that require prolonged Ca^{2+} influx, such as the maintenance of vascular tone and elasticity of arterial walls (35).

Atherosclerosis causes electrophysiological remodeling of VSMC through a replacement of multiple $\text{Ca}_v1.2\alpha1$ variants situ-

Table 1. Comparison of electrophysiological properties of the $\text{Ca}_v1.2\alpha1$ Ca^{2+} channel splice variants identified in VSMC^N ($\alpha_{1C,127}$, $\alpha_{1C,73}$, $\alpha_{1C,125}$, $\alpha_{1C,126}$, and $\alpha_{1C,71}$) and VSMC^D ($\alpha_{1C,77}$) in dependence of β subunits and concentration of charge carriers

α_{1C} isoform	Kinetics of inactivation				Activation		Steady-state inactivation			
	$I_{\max}$, μA (n)	I_o , %	I_f , %	τ_f , ms	$V_{a,0.5}$, mV	k_a (n)	a , %	$V_{i,0.5}$, mV	k_i (n)	
$\alpha_{1C}/\beta_{1a}/\alpha_2^{\delta-1}$, 40 mM Ba^{2+}										
$\alpha_{1C,127}$	-2.70 ± 0.35 (21)	$20.0 \pm 1.3^{\dagger}$	43.6 ± 2.5	$89.7 \pm 7.3^{\dagger}$	0.3 ± 1.8	$9.2 \pm 0.5^*$ (9)	$20.9 \pm 3.8^*$	$-24.4 \pm 2.7^*$	$12.4 \pm 1.0^*$ (8)	
$\alpha_{1C,73}$	-4.36 ± 0.59 (19)	16.6 ± 1.5	49.0 ± 3.7	114.9 ± 10.7	1.8 ± 3.2	$8.8 \pm 0.6^*$ (8)	12.4 ± 5.8	-17.2 ± 4.4	8.1 ± 1.1 (7)	
$\alpha_{1C,125}$	-3.34 ± 0.31 (23)	$11.6 \pm 0.9^*$	51.8 ± 3.6	134.2 ± 10.9	-5.3 ± 1.4	$9.5 \pm 1.0^*$ (8)	$5.7 \pm 1.4^{\S}$	$-21.4 \pm 1.6^*$	$12.1 \pm 1.2^*$ (8)	
$\alpha_{1C,126}$	-3.21 ± 0.52 (18)	$14.9 \pm 0.9^{\S}$	38.5 ± 3.3	133.6 ± 9.1	-1.2 ± 3.0	$8.2 \pm 0.9^*$ (8)	$15.1 \pm 1.3^*$	-11.7 ± 2.4	8.8 ± 1.7 (5)	
$\alpha_{1C,71}$	-4.07 ± 0.43 (26)	16.1 ± 1.5	43.5 ± 3.7	121.1 ± 8.6	0.5 ± 1.7	$7.5 \pm 0.6^*$ (9)	9.1 ± 4.5	$-22.9 \pm 1.7^*$	8.2 ± 1.0 (6)	
$\alpha_{1C,77}$	-4.04 ± 0.68 (38)	18.6 ± 1.1	44.0 ± 2.8	105.7 ± 8.4	0.6 ± 1.0	4.6 ± 0.3 (17)	7.6 ± 1.6	-8.8 ± 0.7	7.8 ± 1.0 (16)	
$\alpha_{1C}/\beta_{1a}/\alpha_2^{\delta-1}$, 40 mM Ca^{2+}										
$\alpha_{1C,127}$	-1.87 ± 0.20 (26)	4.7 ± 0.9	$89.2 \pm 0.5^*$	$30.1 \pm 0.9^*$	11.4 ± 1.7	$12.2 \pm 0.7^*$ (6)	1.6 ± 1.1	-24.3 ± 1.3	$14.1 \pm 0.8^*$ (5)	
$\alpha_{1C,73}$	-2.84 ± 0.52 (15)	$0.5 \pm 1.8^{**}$	$97.0 \pm 0.4^{**}$	$24.4 \pm 1.2^{**}$	7.3 ± 3.7	9.7 ± 0.4 (5)	1.2 ± 0.7	-23.9 ± 3.6	10.8 ± 0.9 (5)	
$\alpha_{1C,125}$	-3.47 ± 0.41 (17)	5.4 ± 0.6	$88.7 \pm 0.5^*$	$36.6 \pm 2.4^*$	3.9 ± 1.7	8.4 ± 0.4 (13)	0.3 ± 0.3	-21.8 ± 3.1	10.9 ± 0.9 (7)	
$\alpha_{1C,126}$	-3.22 ± 0.59 (13)	$3.2 \pm 0.5^{\dagger}$	$89.1 \pm 0.9^*$	$34.1 \pm 1.9^*$	3.5 ± 1.7	9.0 ± 0.2 (5)	1.0 ± 0.5	-18.4 ± 1.4	8.7 ± 0.5 (5)	
$\alpha_{1C,71}$	-3.69 ± 0.40 (20)	$1.6 \pm 1.0^{\dagger}$	$93.5 \pm 1.0^{\dagger}$	$31.4 \pm 2.3^*$	3.3 ± 1.7	8.6 ± 0.5 (9)	1.8 ± 1.3	-17.7 ± 2.8	8.7 ± 0.2 (5)	
$\alpha_{1C,77}$	-3.52 ± 0.80 (28)	4.3 ± 0.6	93.5 ± 0.5	24.6 ± 1.2	9.6 ± 1.7	10.0 ± 0.7 (10)	1.3 ± 0.8	-16.3 ± 2.4	9.0 ± 1.3 (8)	
$\alpha_{1C}/\beta_{2a}/\alpha_2^{\delta-1}$, 2.5 mM Ca^{2+}										
$\alpha_{1C,127}$	-0.46 ± 0.01 (6)	9.6 ± 3.2	80.5 ± 1.2	38.4 ± 1.0	$24.2 \pm 4.9^*$	$10.9 \pm 0.4^*$ (3)	0.1 ± 0.2	-15.4 ± 4.2	$14.0 \pm 1.7^*$ (3)	
$\alpha_{1C,77}$	-1.42 ± 0.38 (10)	13.3 ± 0.2	73.8 ± 2.4	36.0 ± 3.0	0.9 ± 1.8	8.6 ± 0.2 (4)	0.7 ± 0.3	-14.9 ± 5.6	11.8 ± 1.8 (3)	

Values are reported as means $\pm$ SEM. Differences were tested for by ANOVA. $I_{\max}$, maximum amplitude of the current; I_o and I_f , sustained and fast components of the current; $V_{a,0.5}$, midpoint potential of activation; $V_{i,0.5}$, voltage at half-maximum of inactivation; a , fraction of noninactivating component of the current; k_a and k_i , slope factors; n , number of tested oocytes. *, $P < 0.05$ by Dunnett's test using $\alpha_{1C,77}$ as control. For all pairs comparisons, Tukey's test was used: †, $P < 0.05$ vs. $\alpha_{1C,125}$; ‡, $P < 0.05$ vs. $\alpha_{1C,126}$; §, $P < 0.05$ vs. $\alpha_{1C,127}$.

Materials and Methods

Arterial Tissue. Arterial biopsy samples (three carotid and three femoral) were obtained at surgery from six patients of a mean age of 65 years (Table 2). After having received preoperative written informed consent for the protocol approved by the Western Institutional Review Board (Olympia, WA), vascular tissue was obtained intraoperatively during surgical procedures for atherosclerotic vascular disease. Small areas not visually affected by atherosclerosis and those occluded with heavy plaque burden were identified and dissected. Tissue was washed in 4°C saline, immersed in OCT compound (10.24% polyvinyl alcohol/4.26% polyethylene glycol) (Electron Microscopy Sciences, Hatfield, PA) and frozen in liquid nitrogen.

LCM and Isolation of mRNA. Serial cryostat sections (5–7 μm thick) were cut from the frozen tissue samples. Before LCM, VSMC in the sections were fixed in acetone and quickly immunostained with anti-smooth muscle α -actin monoclonal IgG_{2a} (N1584, DAKO, Glostrup, Denmark). LCM was performed with PixCell II system (Arcturus, Sunnyvale, CA) by using a 7.5- μm laser spot. The excised VSMC were captured on LCM Caps. RNA was extracted from the collected cells by using PicoPure RNA isolation kit (Arcturus) and treated with RNase-free DNase (Qiagen, Valencia, CA).

PCR. RT-PCR was carried out with a RETROscript kit (Ambion, Austin, TX) and RNA isolated from 200–300 microdissected cells. To increase specificity of PCR, a second round with nested primers was used. The identified $\text{Ca}_v1.2\alpha1$ variants were subcloned into the Melton's vector. All nucleotide sequences were verified by DNA sequencing.

Real-time PCR was carried out in a GeneAmp 5700 Sequence Detection System (Applied Biosystems, Foster City, CA) by using

the comparative threshold cycle method to determine the $\text{Ca}_v1.2\alpha1$ expression in VSMC^N and VSMC^D of the same patient (*Supporting Materials and Methods*, which is published as supporting information on the PNAS web site).

Electrophysiology. Ba^{2+} and Ca^{2+} currents were recorded in 40 mM Ba^{2+} or Ca^{2+} at 20–22°C by a two-electrode voltage clamp in *Xenopus* oocytes 3 days after microinjection with a mixture of mRNAs coding for a $\text{Ca}_v1.2\alpha1$ variant and auxiliary β_{1a} and $\alpha_2\delta$ -1 subunits (1:1:1, mol/mol). Additional experiments with selected channel isoforms ($\alpha_{1C,77}$ and $\alpha_{1C,127}$) that showed statistically significant differences in electrophysiological properties were carried out by using 2.5 mM Ca^{2+} close to physiological calcium concentration and the primary cardiac β_{2a} subunit (X64297). Inactivation time constants (τ) were determined from the double exponential fitting of the current decay. The activation and steady-state inactivation curves were fitted with Boltzmann equations. All fits were obtained with individual measurements and then averaged.

Statistical Analysis. Results are presented as mean $\pm$ SEM. Differences of the measured electrophysiological parameters were tested for by ANOVA by using Tukey's test for all pair comparisons and by using Dunnett's test when data were compared with $\alpha_{1C,77}$ as control. Probability values of $P < 0.05$ were considered to be statistically significant.

Further methodological details can be found in *Supporting Materials and Methods*.

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