Vasa recta voltage-gated Na\(^{+}\) channel Na\(_{\text{v}}\).1.3 is regulated by calmodulin

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Lee-Kwon W, Goo JH, Zhang Z, Silldorff EP, Pallone TL. Vasa recta voltage-gated Na\(^{+}\) channel Na\(_{\text{v}}\).1.3 is regulated by calmodulin. Am J Physiol Renal Physiol 292: F404–F414, 2007. First published August 15, 2006; doi:10.1152/ajprenal.00070.2006.—Rat descending vasa recta (DVR) express a tetrodotoxin (TTX)-sensitive voltage-operated Na\(^{+}\) (Nav) conductance. We examined expression of Nav isoforms in DVR and tested for regulation of Nav currents by calmodulin (CaM). RT-PCR in isolated permeabilized DVR using degenerate primers targeted to TTX-sensitive isoforms amplified a product whose sequence identified only Nav.1.3. Immunoblot of outer medullary homogenate verified Nav.1.3 expression, and fluorescent immunostaining showed Nav.1.3 expression in isolated vessels. Immunohistochemistry in outer medullary serial sections confirmed that Nav.1.3 is confined to \(\alpha\)-smooth muscle actin-positive vascular bundles. Nav.1.3 possesses a COOH-terminal CaM binding motifs. Using pull-down assays and immunoprecipitation experiments, we verified that CaM binds to either full-length Nav.1.3 or a GST-Nav.1.3 COOH-terminal fusion protein. In patch-clamp experiments, Nav currents were suppressed by calmodulin inhibitory peptide (CIP; 100 nM) or the CaM inhibitor \(\mathrm{N}-(6\text{-aminohexyl})-5\text{-chloro}-1\text{-naphthalene-sulphonamide}\) hydrochloride (W7). Neither CIP nor W7 altered the voltage dependence of pericyte Nav\(_{\text{v}}\) or Nav currents; however, raising electrode free Ca\(^{2+}\) from 20 to \(~2,000\) nM produced a depolarizing shift of activation. In vitro binding of CaM to GST-Nav.1.3C was not affected by \(\mathrm{Ca}^{2+}\) concentration. We conclude that Nav.1.3 is expressed by DVR, binds to CaM, and is regulated by CaM and Ca\(^{2+}\). Inhibition of CaM binding suppresses pericyte Nav\(_{\text{v}}\) currents.

METHODS

Isolation of DVR. Investigations involving animal use were performed according to protocols approved by the Institutional Animal Use and Care Committee of the University of Maryland. Sprague-Dawley rats (120–200 g) were anesthetized by intraperitoneal injection of ketamine (80 mg/kg) and xylazine (10 mg/kg). Under deep anesthesia, the abdomen was opened and the kidneys were excised. Euthanasia was induced by exsanguination under anesthesia, without the rats regaining consciousness. For patch-clamp studies, tissue slices were stored at 4°C in a physiological saline solution (PSS; in mmol/l: 155 NaCl, 5 KCl, 1 MgCl\(_2\), 1 CaCl\(_2\), 10 HEPES, and 10 glucose, pH 7.4). Small wedges of renal medulla were dissected and transferred to BlenDzyme 1 (Roche) at 0.27 mg/ml in high-glucose DMEM media (In vivogen), incubated at 37°C for 30 min, transferred to PSS, and stored at 4°C. At intervals, DVR were isolated from the enzyme-digested renal tissue by hand dissection and transferred to a perfusion chamber for patch-clamp recording. Gigaseals were directly formed on abluminal pericytes of intact vessels as previously illustrated (26).

RT-PCR. As previously described (21), kidneys were perfused with solution A containing (in mmol/l) 135 NaCl, 1 KCl, 0.1 Na\(_{3}\)HPO\(_4\), 0.12 Na\(_{2}\)SO\(_4\), 1.2 MgSO\(_4\), 0.3 NaOAc, 5 HEPES, 2.5 CaCl\(_2\), 1 DTT, and 5.5 glucose, pH adjusted to 7.4 with NaOH. Solutions for reverse transcriptase (RT)-PCR were prepared by adding either vanadyl ribonucleoside complex (VRC; 10 mM, solution B) or RNase inhibitor (Clontech, 1 U/\(\mu\)l, solution C) to solution A. Kidney slices were digested with collagenase type 1 (1 mg/ml), transferred to a petri dish, and maintained at 4°C. DVR and nephrin segments were isolated by microdissection in solution B. Those structures were cleared of debris by transfer to a separate petri dish that contained solution C as a wash.
buffer. The microdissected structures were subsequently transferred from the wash buffer to an RT-PCR reaction tube. Each sample was centrifuged at 10,000 g for 10 min and then rinsed three times with 100 μl of ice-cold solution C. Samples of the wash buffer were also analyzed by RT-PCR to rule out contamination. Those controls were uniformly negative.

Microdissected DVR segments were permeabilized in solution containing 2% Triton X-100, 5 mM DTT, and 1 U/μl RNase inhibitor. The mRNA from permeabilized DVR was subjected to reverse transcription (RT) using Superscript III (RT-PCR kit, Invitrogen) in a 20-μl volume according to the manufacturer’s instructions. DVR were screened for expression of TTX-sensitive NaV channel isoforms using degenerative forward (5’-GTGATGTCCTACAG/TGTG-3’) and reverse (5’-GCCGCTAGTAAG/TGTGGCAAA-3’) primers. Those primers were designed to yield a 367-bp product from TTX-sensitive NaV isoforms. An additional amplification step was performed using nested forward (5’-GGTCTGCTCTCATGTC/GTTTGCG-3’) and reverse (5’-CCAGCC/TTTCACAGC/ATGATAC-3’) primers to generate a 304-bp product. Both sets of primers were based on the published sequence of TTX-sensitive NaV isoforms corresponding to Genebank accession numbers: NM 030875 (NaV1.1), NM 012647 (NaV1.2), NM 013119 (NaV1.3), and NM133289 (NaV1.7). To avoid degenerative forward (5’-GTCATGATCCTCACC/TGTG-3’), reverse (5’-GCCGCTAGTAAG/TGTGGCAAA-3’), and degenerative forward (5’-GTGATGTCCTACAG/TGTG-3’), reverse (5’-CCAGCC/TTTCACAGC/ATGATAC-3’), and degenerative forward (5’-GTCATGATCCTCACC/TGTG-3’), reverse (5’-GCCGCTAGTAAG/TGTGGCAAA-3’), and reverse (5’-CCAGCC/TTTCACAGC/ATGATAC-3’) primers to generate a 304-bp product. Both sets of primers were based on the published sequence of TTX-sensitive NaV isoforms corresponding to Genebank accession numbers: NM 030875 (NaV1.1), NM 012647 (NaV1.2), NM 013119 (NaV1.3), and NM133289 (NaV1.7). To avoid genomic DNA contamination, all primer combinations span introns. TTX-sensitive NaV isoform expression by the above steps was analyzed from multiple microdissected, permeabilized DVR derived from at least two rats. The PCR products were ethanol precipitated, separated by electrophoresis on 2% (wt/vol) agarose gels, stained with ethidium bromide, and photographed. The nested RT-PCR products were subcloned into pCRI-TOPO vector (Invitrogen) and sequenced to determine specific identity of NaV isoform(s).

Plasmid constructs. COOH-terminal NaV1.3 (NaV1.3C, 250 amino acid) PCR products were generated from outer medullary total RNA by RT-PCR using the forward and reverse primers: 5’-TGGTGCTACATGATCCTCACC/TGTG-3’ and reverse (5’-GCCGCTAGTAAG/TGTGGCAAA-3’). The PCR products were digested from multiple microdissected, permeabilized DVR derived from at least two rats. The PCR products were ethanol precipitated, separated by electrophoresis on 2% (wt/vol) agarose gels, stained with ethidium bromide, and photographed. The nested RT-PCR products were subcloned into pCRI-TOPO vector (Invitrogen) and sequenced to determine specific identity of NaV isoform(s).

Expression and purification of GST and His-tagged fusion proteins. NaV1.3C, in pGEX (pGEX-NaV1.3C), and CaM, in pGEX or pET30, was confirmed by sequencing.

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Pull-down assay. To determine binding of GST-NaV1.3C to CaM or GST-CaM to NaV1.3, the tissue lysates from OM and IM (2 mg) were incubated with either GST-NaV1.3C or GST-CaM (3 μg) bead-bound fusion proteins. Bead-bound GST served as negative control. After being washed four times with binding buffer (in mmol/l: 20 Tris-HCl, 120 NaCl, 1 EDTA, protease inhibitor cocktail, pH 7.4) containing 0.1% Triton X-100, the bound proteins were resolved by SDS-PAGE. NaV1.3 and CaM proteins were detected by immunoblotting with anti-CaM (Zymogen) or anti-NaV1.3 antibodies (Alomone). To verify the specificity of the NaV1.3 antibody, immunoblotting was also performed after preabsorption with antigenic peptide.

Immunoprecipitation. Immunoprecipitation was performed with antibody directed against CaM. Tissue samples were homogenized in lysis buffer (10 mmol/l triethanolamine, 250 mmol/l sucrose, protease inhibitor cocktail, pH 7.4) using a Polytron tissue grinder following which the tissue lysates were incubated on ice for 30 min after adding 1% Triton X-100. Cellular debris was removed by centrifugation (20 min, 1,000 g). Lysates were mixed with anti-CaM antibody and rotated at 4°C for 2 h. Immune complexes were precipitated by adding 50 μl of protein AG agarose beads (Roche Diagnostics) prequili-brated in the lysis buffer. After rocking overnight at 4°C, the beads were recovered and washed four times with lysis buffer containing 0.1% Triton X-100. The immune complexes were eluted with 50 μl of Laemmli buffer and fractionated by SDS-PAGE.

Immunofluorescent labeling of isolated DVR. With the use of methods previously described in detail (6, 21), immunofluorescent labeling was performed to verify expression of NaV1.3, 1.3. Labeling of α-smooth muscle actin (SMA) was performed to identify pericytes. Microdissected DVR were transferred onto slides and fixed with 2% paraformaldehyde in 100 mmol/l cacodylate buffer, pH 7.4. The fixed vessels were incubated overnight at 4°C in PBS containing 5% BSA, 0.1% Triton X-100 (PBS/Triton solution), polyclonal rabbit anti-NaV1.3 channel antibody (Alomone Labs, 1:50), and monoclonal mouse anti-SMA (Sigma, 1:500). After four washes with 1× PBS containing 0.1% Triton X-100, the vessels were incubated with Alexa Flour 488 goat anti-rabbit IgG (1:200) and Alexa Fluor 568 goat anti-mouse IgG (1:200, Molecular Probes, Eugene, OR) for 1 h at room temperature. After several washes with PBS/Triton, coverslips were mounted with Vectorshield (Vector Laboratories, Burlingame, CA). To verify the specificity of NaV1.3 and SMA antibodies, negative controls were performed in which those primary antibodies were omitted. Fluorescent images were obtained using a Zeiss LSM410 confocal microscope.

Immunostaining of kidney sections. Rat kidneys were fixed by perfusing them for 2 min in PBS, 5 min in 2% paraformaldehyde, and 2 min in cryoprotectant (10% EDTA, 0.1 mol/l Tris). After the perfusion fixation, kidneys were removed, postfixed overnight in 2% paraformaldehyde in PBS, and embedded in paraffin. Labeling of 50-μm-thick sections was carried out by the indirect immunoperoxidase method (DAKO Cytomation, Carpinteria, CA). The sections were dewaxed and rehydrated. Endogenous peroxidase was blocked by 3% H2O2 for 30 min at room temperature. Nonspecific binding of IgG was prevented by incubating in 50 mmol/l NH4Cl for 30 min, followed by blocking with 5% BSA, 0.05% saponin, and 0.2% gelatin in PBS. Sections were subsequently incubated overnight at 4°C with primary antibody Diluted in PBS supplemented with 5% BSA, 0.1% Triton X-100. After being rinsed three times (PBS, 5% BSA, 0.1% Triton X-100, 10 min), the sections were incubated in horseradish peroxidase-conjugated goat anti-rabbit IgG and horseradish peroxidase-conjugated goat anti-mouse IgG (DAKO K609). To detect horse-

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radial peroxidase, sections were incubated in either 0.1% 3,3-diaminobenzidine (DAB, brown color) or Vector SG (blue color, Vector Laboratories). After being washed, the sections were dehydrated in a graded series of ethanol solutions and embedded in permount (Sigma).

Whole cell patch-clamp recording. Patch pipettes were made from borosilicate glass capillaries (PG5215-4, external diameter 1.5 mm, internal diameter 1.0 mm; World Precision Instruments, Sarasota, FL), using a two-stage vertical pipette puller (Narshige PP-830) and heat polished to a final resistance of 4–8 MΩ. Whole cell-captured patch recording was performed with a CV201AU headstage and Axopatch 200 amplifier (Axon Instruments, Foster City, CA) at room temperature as previously described (26, 46). The following solution designed to achieve intracellular free Ca$^{2+}$ concentration of ~50 nM was used in the electrode (in mmol/l): 115 Cs[methanesulfonate], 18 CsCl, 10 NaCl, 2.35 MgATP, 1 CaCl$_2$, 5 EGTA, 10 HEPES, pH 7.2 adjusted with CsOH. In experiments designed to evaluate the effect of electrode free Ca$^{2+}$ on Nav current, BAPTA [1,2-bis(o-aminophenoxy)ethane-N,N',N'-tetraacetic acid] was used as a chelator in the electrode. BAPTA concentration was 5 mmol/l, and total CaCl$_2$ concentration was either 0.63 mmol/l (~20 nmol/l free Ca$^{2+}$) or 4.7 mmol/l (~2,000 nmol/l free Ca$^{2+}$), respectively. In some experiments, calmodulin inhibitory peptide (CIP; 100 nmol/l, Ac-Arg-Arg-Lys-Trp-Gln-Lys-Thr-Gly-His-Ala-Val-Arg-Ala-Ile-Gly-Arg-Leu-NH$_2$, Calbiochem) was included in the electrode. The extracellular buffer was PSS. To inhibit K$^+$ channels and Cl$^-$ channels, respectively, all measurements were performed in PSS containing ANG II (10 nmol/l) and niflumic acid (100 μmol/l). ANG II suppresses K$^+$ currents but does not augment Nav currents in DVR pericytes (46). Results have been corrected for junction potentials (26).

Statistics. Curve fits were performed with Clampfit 9.2 (Axon Instruments) using Levenberg-Marquardt algorithms. Data are reported as means ± SE. Significance of effects was evaluated with SigmaStat 3.11 (Systat Software) using parametric or nonparametric tests as appropriate. Group comparisons were performed with Student’s t-test (paired or unpaired, as appropriate) or the rank sum test (nonparametric). Comparisons between multiple groups were with one-way ANOVA, repeated-measures ANOVA, or repeated-measures ANOVA on ranks (nonparametric). Post hoc comparisons were performed using Tukey’s or Holm-Sidak tests. $P < 0.05$ was used to reject the null hypothesis.

RESULTS

Identification of Nav1.3 in DVR. We previously showed that the voltage-gated Na$^+$ current in DVR pericytes is exquisitely sensitive to TTX ($K_d = 2.2$ nmol/l) (46). To determine which Nav isoform(s) are expressed in rat DVR, we designed degenerate, nested primers that yield an expected 304-bp product from the cDNA that codes for the four known TTX-sensitive Nav isoforms (Nav1.1, 1.2, 1.3, and 1.7). Based on sequence alignment and comparison of the α-subunits, we selected conserved regions that provide appropriate targets for amplification while spanning introns to avoid amplification of genomic DNA. Hand-dissected DVR were permeabilized and subjected to RT-PCR. As shown in Fig. 1A, lanes 5 and 6, the nested, degenerate primers yielded the anticipated 304-bp DNA fragment(s). PCR products from four rats (3–4 vessels each) were pooled and subcloned. Seven of the subclones were selected for sequencing, all of which identified expression of Nav1.3. Assuming similar efficiency of amplification for the TTX-sensitive Nav isoforms, these findings imply predominance of Nav1.3 message in DVR.

To confirm Nav1.3 isoform expression in the kidney, tissue lysates from rat outer and inner medulla were subjected to immunoblot analysis. An affinity-purified rabbit polyclonal antibody directed against Nav1.3 recognized three distinct bands near ~250 kDa (Fig. 1B). To confirm expression of Nav1.3 in isolated DVR and determine its distribution to pericytes and endothelium, hand-dissected vessels were immunostained for Nav1.3 and the pericyte marker α-SMA. Figure 2 provides an example of individual and merged confocal fluorescent images. Figure 3 shows additional examples of SMA and Nav1.3 staining along with corresponding white-light differential interference contrast (DIC) photomicrographs. In both panels, the arrowheads point to pericytes and asterisks label endothelial cells. Unexpectedly, Nav1.3 immunostaining was present in both endothelium and pericytes. To determine the distribution of Nav1.3 in the renal medulla, immunostaining was also performed on serial tissue sections. Both SMA and Nav1.3 were confined to DVR in vascular bundles (Fig. 4).

Interaction between CaM and Nav1.3. As with other isoforms, Nav1.3 has a COOH-terminal IQ motif, available for binding and interaction with CaM (17, 23, 31). To explore that interaction, several studies were performed. First, GST fusion proteins that include 250 amino acid COOH-terminal Nav1.3 (GST-Nav1.3C) or full-length CaM (GST-CaM) were generated. GST-CaM, but not GST alone, yielded three bands from medullary homogenate near the expected size, ~250 kDa (Fig. 5A). Those bands were reduced by preabsorption with antigenic peptide (Fig. 5A, lane 2 vs. lane 4). Figure 5B shows that GST-CaM but not GST yielded bands from outer and inner medullary homogenates. The GST-Nav1.3C construct, but not GST alone, bound CaM to yield a single band of the expected size (~17 kDa) from outer and inner medullary homogenates (Fig. 5C). Finally, coinmunoprecipitation of CaM and native Nav1.3 was also performed to verify their association in vivo. In that case, immunoblot with anti-Nav1.3 identified two bands from the immunoprecipitates of anti-CaM antibody (Fig. 5D).

Modulation of pericyte Nav current by CaM. Current-voltage characteristics of fast Na$^+$ currents ($I_{Na}$) in DVR pericytes were studied using the pulse protocol defined by the inset in Fig. 6A. Pericytes were depolarized from the holding potential of ~100 mV to values between ~70 and 40 mV in 10-mV increments. Rapidly activating and inactivating inward currents were observed the electrophysiological characteristics of which have been previously analyzed (46). Inclusion of CIP (100 nmol/l) in the electrode significantly reduced peak inward currents over a range of pulse potentials (Fig. 6B). The voltage dependence of inactivation was investigated using the protocol defined in Fig. 6C, inset. Pericytes were held at various conditioning potentials ranging from ~110 to 10 mV for 2 s and then depolarized to ~10 mV. Conditioning potentials greater than about ~90 mV led to decreases in peak $I_{Na}$. Despite the general reduction of inward current amplitude by CIP, there was no shift in its voltage dependence. Fit of the data to the Boltzman equation, $y = y_0/\{1 + \exp[(V_m - V_{1/2})/K]\}$, where $V_m$ is membrane potential, $s = -1$ or +1 for activation or inactivation, respectively, and $V_{1/2}$ and $K$ are constants, is shown in Fig. 6D. A Levenberg-Marquardt parameter search yielded $V_{1/2}$ and slope factor, $K$ of ~72.1 and 8.64 mV for inactivation, respectively, and ~19.2 and 4.91 mV for activation, respectively.

We also tested the effects of the membrane permeant CaM inhibitor W7 on $I_{Na}$ elicited by sequential pulses from ~100 to ~10 mV at 5-s intervals. During pulsation, W7 was introduced...
into the bath at 1, 10, and 100 μmol/l. Inhibition of peak inward current was achieved at 10 and 100 μmol/l (Fig. 7, A and B). Fit of the data to the equation \( I_{Na}/I_{Na0} = 1/(1 + [W7]/K_d) \) yielded a best fit for \( K_d = 8.04 \, \mu\text{mol/l} \). The ability of W7 to inhibit \( I_{Na} \) was slowly reversible. An example of reversibility is provided as a concatenated display of sequential inward currents in Fig. 7C. \( I_{Na} \) was nearly eliminated by W7 (100 μM) and slow reversal occurred over several minutes during washout. To investigate the effect of W7 on the voltage dependence of activation and inactivation, we used W7 near its \( K_d \), at 10 μmol/l. Higher W7 concentrations eliminated \( I_{Na} \) to a degree that analysis of voltage dependence was impossible. As with CIP (Fig. 6), W7 reduced \( I_{Na} \) without shifting the voltage dependence of activation or inactivation (Fig. 8).

In addition to the COOH-terminal motifs that bind CaM, NaV isoforms possess EF-hand domains that might bind Ca\(^2+\). Thus cytoplasmic Ca\(^2+\) might modify conductance or voltage dependence of gating through EF-hand or CaM interaction. To test whether cytoplasmic Ca\(^2+\) affects NaV current, we measured \( I_{Na} \) in a series of cells, alternating between electrode buffer with low (~20 nmol/l) or high (~2,000 nmol/l) free Ca\(^2+\). In contrast to the effects of interfering with CaM binding (Fig. 6), raising cytoplasmic Ca\(^2+\) shifted the voltage dependence of activation (Fig. 9, A and B). In vitro binding of His-CaM to GST-NaV1.3C was Ca\(^2+\) dependent (Fig. 9C) so that it is unlikely that effects of Ca\(^2+\) on activation of \( I_{Na} \) involve dissociation of CaM from the COOH terminus.

**DISCUSSION**

DVR supply blood flow to the medulla of the kidney. They arise from juxtamedullary glomerular effenter arterioles in the outer stripe of the outer medulla. In the inner stripe, DVR coalesce to join vascular bundles wherein they closely associate with ascending vasa recta and descending thin limbs of short looped nephrons (22). In addition to transmural transport functions that accommodate countercurrent trapping of NaCl and urea, DVR are contractile (28, 29). Pericytes surround the DVR endothelial monolayer and respond to a variety of agents with vasoconstriction and dilation (27). It has been inferred from the anatomical arrangement of DVR in vascular bundles that they regulate both total blood flow to the medulla and its distribution between the outer and inner medulla of the kidney.

In the past, the presence of voltage-gated cation channels in the effenter circulation of the kidney was controversial. More recently, it has been recognized that voltage-gated Ca\(^2+\) channels (Ca\(V\)) are present in the juxtamedullary efferter arterioles and DVR (15, 16, 48). In a recent communication, we reported the surprising finding that voltage clamp depolarizations of DVR pericytes, designed to elicit Ca\(V\) currents, yielded a rapidly inactivating, TTX-sensitive Na\(V\) current. The expected L-type Ca\(V\) currents were only observed when pericytes were treated with the agonist, FPL-64176 (46). In this study, we sought to identify expression of molecular isoforms of Na\(V\) in DVR. RT-PCR amplification of RNA from isolated, perme-
abilized DVR with degenerate primers targeted to TTX-sensitive NaV isoforms yielded only NaV1.3 sequences from hand-dissected vessels. Immunoblot of medullary homogenate (Fig. 1B), immunohistochemistry in isolated vessels (Figs. 2 and 3), and immunohistochemistry in tissue sections (Fig. 4) verified expression of NaV1.3, confined to DVR of outer medullary vascular bundles. To firmly establish that NaV1.3 carries the DVR pericyte fast Na⁺ currents, it would be necessary to silence its expression. Measurements of NaV currents in DVR pericytes have been performed on acutely isolated vessels that are not amenable to long-term incubations required for mRNA silencing. An appropriate cell culture model of the DVR pericyte does not exist. Finally, a murine knockout of NaV1.3 is unavailable and would seem unlikely to be viable.

Beginning with the report of TTX-resistant NaV currents in the vasculature by Sturek and Hermensmeyer (37), smooth muscle NaV have been intermittently described. Mesenteric myocytes have been shown to have a TTX-sensitive NaV conductance (2), while the TTX-resistant skeletal muscle isoform NaV1.5 may be prevalent in gastrointestinal smooth muscle.

Fig. 3. Immunocytochemical detection of NaV1.3 in pericytes and endothelium. Hand-dissected DVR were subjected to immunofluorescence staining. Figure shows high-magnification confocal images of SMA (red) and NaV1.3 (green) fluorescence. Left and right, bottom: white light/differential interference contrast (DIC) images of the same vessels. Endothelial cells (*) that underlie pericytes (arrowheads) show expression of NaV1.3 but not SMA. The results are representative of 7 experiments.

Fig. 4. Localization of NaV1.3 expression in the renal outer medulla. Light micrographs of 50-μm-thick sections illustrate NaV1.3 immunostaining in the renal outer medulla (brown). A and B: positive immunostaining for NaV1.3 protein in outer medullary vascular bundles (brown). C and D: sequential sections corresponding to panels stained for NaV1.3 were positive for α-SMA in vascular bundles (blue). Immunostaining was not detected when the primary antibody was omitted (not shown). The results are similar to sections from 3 rats.
(24, 36, 44). Ten isoforms of voltage-operated Na\(^+\) channels have been identified, nine of which are reasonably placed into a single family (Na\(_V\).1.1-Na\(_V\).1.9), while one other (Na\(_X\)) has more divergent structure and properties (7, 8, 30). Six of the Na\(_V\) isoforms are TTX sensitive while Na\(_V\).1.5, 1.8, 1.9, and Na\(_X\) are resistant (25). A recent report of Na\(_V\).1.6, Na\(_V\).1.7, and Na\(_X\) expression in portal vein myocytes has been provided (32). The DWR pericyte Na\(^+\) conductance is exquisitely TTX sensitive (K\(_d\) = 2.2 nM), and its putative identification as Na\(_V\).1.3 is consistent with that characteristic. It seems likely that Na\(_V\).1.3 is the predominant isoform in DWR because screening of several PCR products yielded only Na\(_V\).1.3 sequences. None the less, it remains possible that other Na\(_V\) isoforms are expressed at lower levels. Significant expression of TTX-insensitive channels in DWR pericytes seems unlikely because 10 nM TTX eliminated inward Na\(^+\) currents in patch-clamp studies (46). Due to their luminal location, DWR endothelium cannot be easily accessed for patch clamp to test for fast Na\(^+\) currents and examine TTX sensitivity. Appropriate cell culture models of either DWR pericytes or endothelium do not exist to facilitate electrophysiological studies. Since the degenerate primers we used would not identify TTX-resistant isoforms, it remains possible that the DWR endothelium expresses Na\(_V\).1.5, 1.8, 1.9, or Na\(_X\).

The isoform-specific Na\(_V\).1.3 antibody used in these studies recognized three protein bands, either from medullary homogenate (Fig. 1B), or isolated with GST-CaM binding studies (Fig. 5, A and B). The specificity of antibody recognition was verified though preabsorption with the immunizing antigen. The associated epitope of the commercial antibody corresponds to amino acids 511–524 of the intracellular loop between domains I and II (HLEGHRAGDRFP, acquisition P08104; Fig. 5A). Nav are large molecules comprised of four homologous domains (I-IV) each characterized by six (S1-S6) \(\alpha\)-helical membrane-spanning regions. Splice variants of Na\(_V\).1.3 alter the intracellular loop between domains I and II through alternate splicing of exon 12. The known splice variants do not affect the antibody binding site. Three and four splice variants of Na\(_V\).1.3 have been described in rat and human, respectively (14, 33, 38). The three rat variants differ from the full-length Nav1.3 described in humans (2,000 amino acids, acquisition number NM_006922.2) by deletion of 51, 138, or 147 nucleotides (38). Those splice variants are expected to yield proteins of 1951, 1954, or 1983 amino acids, respectively. Given that the former two should comigrate during SDS-PAGE, it is unlikely that they correspond to the two lower molecular weight bands in Figs. 1 and 5A. It is possible that the middle band is a previously unknown splice variant that exists in the rat kidney or that a posttranslational modification of a known Na\(_V\).1.3 splice variant accounts for its presence. The coprecipitation of CaM (Fig. 5D) yielded two bands recognized by the antibody. The reason for the absence of the middle band in the CaM coprecipitation is uncertain, although expression of the known splice variants of Na\(_V\).1.3 in the rat could account for the pattern in Fig. 5D. The apparent increase in association of Na\(_V\).1.3 with CaM in inner vs. outer medullary homogenate shown in Fig. 5D is also of interest, suggesting enhanced association of these proteins through stabilized interaction in the vasculature of the inner medulla compared with the outer medulla. Given that the function of Na\(_V\).1.3 in pericytes and endothelium remains to be fully elucidated, we cannot provide a sound hypothesis for the purpose of that difference.

The COOH terminus of voltage-gated Na\(^+\) channels express consensus IQ and Baa motifs for interaction with CaM. Mori et al. (23) showed that binding of CaM to those sites was Ca\(^{2+}\)
independent and dependent, respectively. We examined the presence of CaM binding and its modulation of DVR NaV currents. GST fusion constructs of NaV1.3 COOH terminus (GST-NaV1.3C) and CaM were generated to perform pull-down assays. Those experiments verified that GST-NaV1.3C binds to CaM (Fig. 5C). Conversely, GST-CaM binds to native NaV1.3 from medullary lysates (Fig. 5, A and B). Finally, antibody directed against CaM coprecipitates Na V1.3 (Fig. 5D). Studies by Herzog and colleagues (17) examined the effect of COOH-terminal deletions from the TTX-sensitive isoform, NaV1.4. In their hands, deletions that included the IQ motif virtually eliminated currents from transfected cells. Similarly, mutations of the IQ motif of NaV1.6 led to marked reduction of current density. Those results mirror our findings that blockade of CaM interactions using CIP or W7 reduced native NaV currents in DVR pericytes (Figs. 6 – 8). The Ca\textsuperscript{2+}/H\textsuperscript{11001} independence of CaM binding to GST-NaV1.3C (Fig. 9C), combined with the inhibitory effects of CIP support the interpretation that CaM exerts permissive effects on DVR NaV1.3 channel activity through Ca\textsuperscript{2+}-independent binding to the IQ motif. Moreover, as with mutation of the IQ motif of NaV1.4 (17), the voltage dependence of activation and inactivation of \( I_{\text{Na}} \) in DVR pericytes was not affected by CIP or W7 (Figs. 6 and 8).

In contrast to inhibition of CaM binding by CIP (Fig. 6), modulating cytoplasmic Ca\textsuperscript{2+} concentration of pericytes through BAPTA chelation within the patch-clamp electrode induced a significant change in the voltage dependence of NaV current activation. Compared with 20 nM free Ca\textsuperscript{2+}, ~2,000 nM free Ca\textsuperscript{2+} caused a depolarizing shift (Fig. 9, A and B). Parallel in vitro binding studies of the effects of free Ca\textsuperscript{2+} on the binding of CaM to GST-NaV1.3C showed no effect, strongly suggesting that the depolarizing shift does not require dissociation of CaM from the NaV1.3 COOH terminus (Fig. 9C). CaM has a bilobed structure with two EF-hand domains at each of its NH\textsubscript{2} and COOH termini. Association of CaM with its binding partners may be Ca\textsuperscript{2+}/H\textsuperscript{11001} dependent or independent. The modulatory effects of Ca\textsuperscript{2+}/H\textsuperscript{11001} on the function of interacting proteins can also be dependent on or independent of Ca\textsuperscript{2+}/H\textsuperscript{11001} binding to the EF-hand domain that exists on COOH termini (34, 43). Given the complex array of possible interactions, we cannot be certain whether the Ca\textsuperscript{2+}-dependent shift in voltage dependence of activation (Fig. 9, A and B) is mediated through CaM. It seems unlikely, however, that the mechanism involves dissociation of CaM from NaV1.3 (Fig. 9C).
The immunochemical finding that NaV1.3 is expressed by the DVR endothelium (Figs. 2 and 3) is unexpected but not unique (12, 13, 39, 41). Gordienko and Tsukahara (12) observed TTX-resistant NaV currents in cultured endothelia from rat interlobar arteries and human umbilical vein. Similarly, TTX-resistant currents were identified in cultured human saphenous vein endothelia. In that study, RT-PCR amplified a product from the 3'-untranslated region of hH1 (NaV1.5). Expression level was dependent on the source of human serum but immunochemistry identified clear staining in sections from native tissue, mitigating against an artifact related to cell culture (13). In addition to TTX-resistant isoforms, TTX-sensitive NaV have been identified in cultured microvascular endothelial cells from cardiac ventricle (41) and bovine aorta (39). Interestingly, the latter study found that elimination of Na+ influx by TTX or by extracellular Na+ replacement with N-methyl-D-glucamine inhibited extracellular signal related kinase (ERK1/2) activation by shear stress. The manner in which voltage-gated Na+ influx participates in ERK1/2 activation is unknown. We have shown that stretch-related responses occur in DVR endothelium (47) so that a role for endothelial Nav1.3 expression in stretch-activated signal transduction will be a topic worthy of future exploration.

The functional role(s) of Nav1.3 expression in DVR is uncertain. Similarly, the role of NaV expression in various types of smooth muscle is controversial. In neurons, coordinated opening of Nav channels provides a depolarizing Na+ current that facilitates transmission of neural impulses and release of neurotransmitters. DVR endothelia are an electrical syncytium connected through gap junctions (45). Based on that finding, a possibility is that NaV participate in the conduction of depolarizing waves along the vessel axis. It has been proposed that Na+ entry into smooth muscle increases the rate of depolarization to stimulate opening of voltage-gated Ca2+ channels. Such activity might serve to shape the spatial or temporal profile of cytoplasmic Ca2+ concentration changes within the cell that confer specificity to Ca2+ signaling (3, 5).

An alternate possibility is that resting membrane potential lies within the range at which NaV are neither completely activated nor inactivated. The associated small NaV "window current" might provide a steady trickle of Na+ ions into the cell the magnitude of which is regulated through subtle variation of membrane potential. Similarly, the intracellular loop between domains I and II has consensus sites for PKA, PKC, and casein kinase II so that regulation of a putative window current by kinase cascades is possible. Entry of Na+ into cells is energetically costly unless it is utilized as a surrogate Ca2+ entry mechanism via Na+/Ca2+ exchange (1, 4). If NaV and Na+/Ca2+ exchangers exist in sequestered cellular “microdomains,” substantial near membrane elevations of Ca2+ concentration might occur to influence nearby signaling events or to load Ca2+ into cellular stores. Experimentally, such near membrane
cytoplasmic Ca\textsuperscript{2+} changes might be difficult to observe with conventional fluorescent probes that have high Ca\textsuperscript{2+} affinity and distribute diffusely into the cytoplasm. Given the fact that Na\textsubscript{V} expression has been observed in nonexcitable cells as diverse as chondrocytes (11, 40) and corneal epithelium (10, 42), it seems likely that some roles of Na\textsubscript{V} are unknown. The beta subunits associated with Na\textsubscript{V} behave as cell adhesion molecules so that a role of Na\textsubscript{V} in cell matrix interactions has been proposed (19).

Fig. 8. Inhibition of Na\textsubscript{V} currents by calmodulin inhibitor, W7. A and B: examples of I-V currents elicited by pulse depolarizations using the protocol defined by Fig. 7A, inset. Protocols were executed in the absence (A) and presence (B) of W7 (10 \textmu mol/l). C: summary of means ± SE of maximal inward currents elicited in the absence (n = 8) and presence (n = 8) of W7 (10 \textmu mol/l). *P < 0.05 vs. 0.10 \textmu mol/l W7. D: superimposed inactivation (g\textsubscript{Na}/g\textsubscript{Na,max}, left ordinate) and activation (g\textsubscript{Na} /g\textsubscript{Na,max}, right ordinate) plots for DVR pericyte Na\textsubscript{V} conductance. Voltage dependence of activation (n = 8, 8) and inactivation (n = 7, 8) of controls and W7-treated cells was indistinguishable. Curves and insets show best fit of averaged data to Boltzman equations.

Fig. 9. Modulation of Na\textsubscript{V} currents and CaM binding by free Ca\textsuperscript{2+}. A: maximum inward current vs. pulse potential using BAPTA-chelated free Ca\textsuperscript{2+} ([Ca\textsuperscript{2+}]\textsubscript{f}) in the electrode buffer of 20 nM (●) or 2,000 nM (○; n = 6 each; *P < 0.05). B: activation (g\textsubscript{Na}/g\textsubscript{Na,max}) of Na\textsubscript{V} conductance vs. pulse potential in 20 or 2,000 nM [Ca\textsuperscript{2+}]\textsubscript{f} (*P < 0.05). Curves and insets show best fit of averaged data to Boltzman equations. C: measurement of in vitro binding of CaM to GST-Na\textsubscript{V}1.3C in buffers. Purified His-CaM was incubated with immobilized GST-Na\textsubscript{V}1.3C in the presence of various free Ca\textsuperscript{2+} concentrations, set with Ca\textsuperscript{2+}/EGTA buffers. The amounts of His-CaM bound to GST-Na\textsubscript{V}1.3C were determined by Western blotting with anti-CaM antibody. Similar to n = 3 experiments.
In summary, we used RT-PCR with degenerate primers for TTX-sensitive voltage-operated Na⁺ channels to amplify a product from DVR whose sequence identified Nav.1.3 expression. Immunocytochemistry showed that both DVR pericytes and endothelium express Nav.1.3. Reciprocal pull-down assays with GST fusion proteins of COOH-terminal Nav.1.3 or CaM verified their ability to, respectively, bind CaM or Nav.1.3 from inner and outer medullary homogenates. Inhibition of CaM binding with an interfering peptide or block of CaM with the membrane permeant inhibitor W7 reduced DVR pericyte Nav currents and that CaM, is essential for pericyte Nav currents. We speculate that Nav currents in DVR may serve to shape the spatial and temporal profile of Ca²⁺ signaling events by stimulating Cav current or through Na⁺/Ca²⁺ exchange.

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