Coexpression of Voltage-Dependent Calcium Channels Ca\textsubscript{1.2}, 2.1a, and 2.1b in Vascular Myocytes

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Abstract—Voltage-dependent Ca\textsuperscript{2+} channels Ca\textsubscript{1.2} (L type) and Ca\textsubscript{2.1} (P/Q type) are expressed in vascular smooth muscle cells (VSMCs) and are important for the contraction of renal resistance vessels. In the present study we examined whether native renal VSMCs coexpress L-, P-, and Q-type Ca\textsuperscript{2+} currents. The expression of both Ca\textsubscript{2.1a} (P-type) and Ca\textsubscript{2.1b} (Q-type) mRNA was demonstrated by RT-PCR in renal preglomerular vessels from rats and mice. Immunolabeling was performed on A7r5 cells, renal cryosections, and freshly isolated renal VSMCs with anti-Ca\textsubscript{1.2} and anti-Ca\textsubscript{2.1} antibodies. Conventional and confocal microscopy revealed expression of both channels in all of the smooth muscle cells. Whole-cell patch clamp on single preglomerular VSMCs from mice showed L-, P-, and Q-type currents. Blockade of the L-type currents by calciseptine (20 nmol/L) inhibited 35.6±3.9% of the voltage-dependent Ca\textsuperscript{2+} current, and blocking P-type currents (\textomega-agatoxin IVA 10 nmol/L) led to 20.2±3.0% inhibition, whereas 300 nmol/L of \textomega-agatoxin IVA (blocking P/Q-type) inhibited 45.0±7.3%. In rat aortic smooth muscle cells (A7r5), blockade of L-type channels resulted in 28.5±6.1% inhibition, simultaneous blockade of L-type and P-type channels inhibited 58.0±11.8%, and simultaneous inhibition of L-, P-, and Q-type channels led to blockade (88.7±5.6%) of the Ca\textsuperscript{2+} current. We conclude that aortic and renal preglomerular smooth muscle cells express L-, P-, and Q-type voltage-dependent Ca\textsuperscript{2+} channels in the rat and mouse. (Hypertension. 2006;47:735-741.)

Key Words: kidney ■ muscle, smooth ■ calcium

Voltage-dependent Ca\textsuperscript{2+} influx in vascular smooth muscle cells (VSMCs) involves activation of high-voltage–activated (L-type) and in some cases low-voltage–activated (T-type), voltage-dependent Ca\textsuperscript{2+} channels (Ca.s). The distribution of Ca\textsubscript{s} is segment specific: the smallest mesenteric arterioles express T-type Ca\textsubscript{s}, whereas larger mesenteric arterioles express both L-type and T-type Ca\textsubscript{s}. Also, in renal preglomerular vasculature, the distribution of L-type and T-type Ca\textsuperscript{2+} channels may be heterogeneous. In addition to L- and T-type channels, vascular smooth muscles from a number of sources express P/Q-type channels that have functional importance in renal afferent arterioles.

P- and Q-type channels are splice variants of a single gene, Ca\textsubscript{2.1} (Ca\textsubscript{2.1a} and Ca\textsubscript{2.1b}, respectively), and they are distinguished pharmacologically by their sensitivity to \textomega-agatoxin IVA. This difference in sensitivity is attributed to the insertion of 2 amino acids (Asp\textsubscript{Val}Pro\textsubscript{Val}) in the extracellular IVS3-IVS4 linker of the Ca\textsubscript{2.1b} (Q-type) splice variant. A slower inactivation of the Q-type channel compared with the P-type has been ascribed to the insertion of 1 amino acid (Val\textsubscript{Asp}) in the I-II linker region of Ca\textsubscript{2.1b}.

It is not known whether both P and Q channels are expressed in native smooth muscle or whether they are coexpressed with each other and with L-type channels. Moreover, P- and Q-type Ca\textsuperscript{2+} currents have not been shown in native vascular smooth muscle despite the inhibitory effect of P/Q channel blockers on vascular contraction in the renal vascular bed.

The main purpose of the present study was to determine whether VSMCs express both P- and Q-type channels, and if so, whether they contribute to Ca\textsuperscript{2+} currents. We used an RT-PCR–based strategy to distinguish between the splice variants Ca\textsubscript{2.1a} and Ca\textsubscript{2.1b} in renal resistance vessels. In addition, we modified the procedure of Loutzenhiser and Loutzenhiser for isolation of vascular smooth muscle from renal preglomerular vessels from rats to isolation of mouse smooth muscle cells. Single smooth muscle cells were subjected to whole cell patch clamp investigation, and distinct Ca\textsuperscript{2+} currents were identified based on the application of channel subtype–specific toxins. The coexpression of several subtypes of Ca\textsubscript{s} in single smooth muscle cells was validated by immunostaining and confocal microscopy.

Methods

Animals

Animal care followed the guidelines of the National Institutes of Health, and permissions were obtained from the Danish Animal Experiments Inspectorate under the Danish Ministry of Justice. Male Sprague-Dawley rats and C57BL/6 mice had free access to rodent chow and tap water.
RT-PCR and Ribonuclease Protection Assay

Preglomerular vessels were dissected from rats and mice, and RNA was extracted as described previously. RNA aliquots from the rat kidney and kidney regions were from an experimental series used previously and characterized. RT-PCR was performed with an annealing temperature of 50°C in 32 cycles. The primers included: forward primer, CaV1.2: 5′-ccagcagacagacagg-3′; reverse: 5′-ccacacacaaagcc-3′, covering bases 4777 to 4994 (GenBank Access No. NM007971; mouse); forward primer, CaV-2.1: 5′-gctcgtgtgtaaca-3′; reverse, CaV-2.1a: 5′-ctaggtagatgatcttc-3′, covering bases 4642 to 4826 (185 bp rat) and 4498 to 4682 (mouse); and CaV-2.1b: 5′-caggtaggtgatcattgctc-3′, covering bases 4642 to 4824 +6 bp, 189 bp (GenBank accession no. NM04373 for rat and NM007578 for mouse). The CaV-2.1–CaV-2.1a PCR product was cloned in plasmid pSP73, sequenced, and linearized as described. Subsequent in vitro transcription gave a radiolabeled antisense RNA probe discriminating between CaV-2.1a and b. Thus, hybridization and subsequent RNase digestion would protect a 185-bp product for CaV-2.1a (P type) and a 165-bp product for CaV-2.1b (Q type). Ribonuclease protection was performed exactly as described previously.

Cells

Preglomerular smooth muscle cells from mice were isolated using a protocol modified from Loutzenhiser and Loutzenhiser. C57BL/6 mice were anesthetized by ketamine (100 mg/kg) and xylazine (10 mg/kg), and the aorta was catheterized for perfusion of the kidneys. In vivo perfusion was performed with a 3.5% agarose solution (Sea prep, Cambrex) at 37°C. The kidneys were removed and chilled on ice to solidify the agarose. Cortical slices were incubated in isolation medium containing collagenase NB8, 0.5 g/L (Serva); trypsin inhibitor, 0.1 g/L (Sigma); dispase II, 0.35 g/L (Roche); and DNase I, 1.8 mL/L (Roche), for 45 minutes at 37°C. Interlobular arteries and afferent arterioles were collected under a microscope and transferred to medium containing collagenase NB8, 0.5 g/L (Serva); elastase, 13 U/mL (Worthington); protease, 1.5 g/L (Sigma); collagenase NB8, 1.5 g/L (Serva); and collagenase, CP, 1.5 g/L (Calbiochem), and incubated 15 minutes at room temperature. Single smooth muscle cells were dispersed mechanically and kept at 4°C until further use. The isolation medium had the following composition (mM): NaCl, 103; KCl, 5.4; NaHCO3, 4.0; NaH2PO4, 1.5; MgSO4, 1.5; MgSO4, 0.8; glucose, 5.1; Na-pyrovate, 0.9; isethionic acid, 30; HEPES, 5.6; and (in ml/L): MEM vitamin solution (Sigma M6895), 10; MEM essential amino acid solution (Sigma M5550), 20; and MEM nonessential amino acid solution (Sigma M7145), 10.

Renal VSMCs from rats were isolated as described by Gordienko et al. Renal vessels (interlobar, arcuate, and interlobular arteries) were in physiological salt solution (PSS) (in mmo/L: 120 NaCl, 6 KCl, 12 glucose, 10 HEPES, 1.2 MgCl2, and 2.5 CaCl2) on ice; transferred to Ca2+-free PSS (CaCl2 replaced by MgCl2); cut into 1-mm pieces; incubated with 1 mM bovine albumin fraction V (Sigma), collagenase type 1 (Sigma), and trypsin inhibitor type II-S (Sigma) and 0.8 U/mL elastase type 1 (Sigma) at 37°C for 40 minutes; rinsed in ice-cold Ca2+-free PSS; triturated with a modified, wide-bore Pasteur pipette; and 1/3 vol of PSS was added.

Immunohistochemistry

Cryosections (5 μm) from rat kidneys were permeabilized in methanol and blocked with 5% goat serum +1% BSA in Tween Tris buffer saline (TTBS). Primary antibody was rabbit anti-αS, or rabbit anti-αS antibody (Alomone Labs, Jerusalem, Israel). Secondary antibody was horseradish peroxidase–conjugated goat anti-rabbit IgG (DAKO) and Alexa 488– or Alexa 568–conjugated goat anti-rabbit IgG (Molecular Probes), all diluted 1:1000 in TTBS. Horseradish peroxidase–labeled specimens were stained with diamino-
benzidine (DAB) (DAKO) and counterstained with hematoxylin. Images were captured with an Olympus BX51 microscope equipped with an HBO fluorescence lamp, WIG and WIBA filters, and an Olympus DP50 digital camera (DP-Soft, Olympus).

Confocal Microscopy

The specimens were mounted on a Leica DM IRBE upright microscope equipped with ×40/1.0 numerical aperture (n.a.) and ×100/1.4 n.a. oil immersion lenses (Leica oil 58884, refractive index of oil extraordinary at 23°C = 1.518, abbe number = 46). Leica TCS 4D confocal attachments, and an air-cooled Argon/Krypton laser. Images were obtained using a 488-nm excitation wavelength and 510-nm long-pass filter, from an average of 4 line scans of ~1-s duration.

Patch Clamp

Patch clamp on isolated preglomerular smooth muscle cells from mice and A7r5 cells in the whole-cell configuration was performed as described. Experiments were performed at room temperature and with a pipette solution at pH 7.38. Immediately after the whole-cell configuration was obtained, the cells were superfused with a solution that facilitated currents through CaV, using Ba2+ as a charge carrier, and the I-V relationship was monitored by the response to 8 voltage steps of 10 mV (range, ~40 to 30 mV) for 400 ms from a holding potential of ~60 mV. Then a repetitive pulse was applied every 5 s (from ~60 mV to 10 mV for 40 ms). After stabilization of the inwardly directed current, the cells were superfused with the same solution now containing calciseptine (20 mmol/L, L-type), α-agatoxin IVA (10 mmol/L, P-type), or α-agatoxin IVA (300 mmol/L, P- and Q-type; Alomone Laboratory).

Statistical Analysis

Data are presented as mean±SE. The significance of changes was calculated by paired t test with Bonferroni reduction for multiple comparisons. P<0.05 was considered significant.

Results

RT-PCR Analysis of CaV-2.1a and CaV-2.1b Splice Variants

To detect whether both CaV-2.1 splice variants were expressed in rat VSMCs, a dual set of PCR primers was designed to distinguish between CaV-2.1a (P-type) and CaV-2.1b (Q-type) at the splice site. The 3′-end of the CaV-2.1a primer covered the splice site such that amplification of the CaV-2.1b splice variant was highly unlikely (Figure 1A). The 3′-end of the CaV-2.1b primer was within the 6-bp insert, allowing amplification only of this variant. RT-PCR assays using these primers were run on RNA extracted from preglomerular renal vessels and with renal cortex as a positive control. Amplification yielded products for both splice variants (Figure 1B), and sequencing of these revealed 100% homology to the respective splice variant sequences. Whether the splicing of CaV-2.1 is the same for rats and mice is uncertain but likely. When cDNA from mouse preglomerular vessels was subjected to PCR amplification products of the expected size were obtained for CaV-1.2, CaV-2.1a, and CaV-2b (Figure 1C). No amplification was observed with water instead of cDNA or when reverse transcriptase was omitted in the RT-PCR, thus confirming the mRNA origin of the amplification products. Ribonuclease protection assay with a labeled riboprobe generated from the cloned PCR product yielded 2 distinct hybridization products with the expected molecular size when whole-kidney RNA was analyzed (Figure 1D). Thus, both P and Q splice variants are expressed in rat kidney. The lowest abundance of both CaV-2.1a and CaV-2.1b was found in the outer medulla, whereas cortex and inner medulla were comparable (Figure 1D). The glyceraldehyde-3-dehydrogenase
mRNA level was not different between kidney regions on the same RNA samples.8

**Immunolabeling of Kidney Sections and Single VSMC for Ca\textsuperscript{2+} Channels**

Next, the distribution of L- and P/Q-type channels was examined along the microvasculature. To this end, we used rat kidney cryosections immunolabeled with either CaV\textsubscript{1.2} or CaV\textsubscript{2.1} antibody. Using DAB chromogen as a substrate for visualization, labeling was associated with the entire renal preglomerular vasculature. Figure 2A and 2B shows staining of cross-sectioned cortical radial arteries branching into afferent arterioles. In separate experiments, immunofluorescence microscopy was performed. This method gave a higher resolution than DAB staining, allowing for the distinction of individual smooth muscle cells. Serial sections revealed positive staining of the same glomerular arterioles for both CaV\textsubscript{1.2} and CaV\textsubscript{2.1} visualized with immunofluorescence (Figure 2C and 2D). Preglomerular arteries were positively labeled for both channels (Figure 2E and 2F). No gaps from unlabeled smooth...
muscle cells appeared in any of the arteries or arterioles. This result strongly indicates that both channel proteins are expressed in all of the preglomerular renal VSMCs. In the preparation of single VSMCs, vessel fragments were left with an intact layer of smooth muscle cells. In 1 such vessel labeled with anti-CaV1.2 antibody, all of the cells displayed fluorescence, and confocal imaging showed that labeling was associated with the plasma membranes (Figure 2G). The immunofluorescence signal for CaV2.1 was more evenly distributed within single smooth muscle and associated with all smooth muscle cells (Figure 2H). To additionally study subcellular localization, confocal microscopy was performed on individual cells. For CaV1.2 there was a higher density of the channel labeling associated with the cell membrane (Figure 2I). CaV2.1 showed a more diffuse labeling throughout the cytoplasm (Figure 2J). Both CaV1.2 and CaV2.1 immunoreactive protein was also found in A7r5 cells (data not shown).

**Patch/Clamp Experiments**

A distinction between different Ca\(^{2+}\) channel subtypes at the functional level was obtained through patch-clamp experiments on freshly isolated mouse preglomerular myocytes (Figure 3A). The cells exhibited typical voltage-activated Ca\(^{2+}\) currents that displayed significant rundown after 5 to 6 minutes. All of the inhibitor experiments were, therefore, done before this time. A typical example of an I-V curve is shown in Figure 3B. The L-type specific blocker calciseptine (20 nmol/L) inhibited the inward current by 35.6±3.9% (n=4); \(\omega\)-agatoxin IVA (10 nmol/L), which blocks P-type currents, inhibited the inward current by 20.2±3.0% (n=4); and, finally, 300 nmol/L \(\omega\)-agatoxin IVA blocking both P- and Q-type currents inhibited the current by 45.0±7.3% (n=3; \(P<0.05\), all, compared with basal; Figure 3C). The current density in preglomerular smooth muscle was 6.03±0.60 pA/pF (SE, n=11). Voltage-activated Ca\(^{2+}\) currents were also found in the aortic smooth muscle cell line (A7r5) where rundown was observed after 6 to 8 minutes. When 20 nmol/L of calciseptine was used, the Ca\(^{2+}\) current was inhibited by 30.6±2.8% (n=5; Figure 4). The next question was whether the effect of the blockers was additive. Because of the fast rundown in mouse preglomerular smooth muscle cells, the following experiments were only performed in A7r5 cells. The current density in A7r5 cells was 1.3±0.2 pA/pF (n=22). Cells were superfused first in the absence of blockers, then with the addition of 20 nmol/L calciseptine. After current measurements with this...
blocker alone, the same cell was superfused with a mixture of 20 nmol/L calcisepine and 10 nmol/L ω-agatoxin IVA. Calcisepine alone inhibited Ca²⁺ current by 28.5±6.1% (as in the previous series), whereas the mixture resulted in an additional reduction of the current by 58.0±11.8% (n=5). Thus, the effects of inhibition of L- and P-type Ca²⁺ channels were additive. In a third series of patch/clamp experiments, cells were first superfused with the superfusion solution alone, then the mixture of 20 nmol/L calcisepine and 10 nmol/L ω-agatoxin IVA, which gave an inhibition corresponding to what had been determined previously (43.2%±2.8, n=5). Subsequently, the same cell was superfused with a mixture of 20 nmol/L calcisepine and 300 nmol/L ω-agatoxin IVA, which should block L-, P-, and Q-type currents. This procedure resulted in inhibition of 88.7±5.6% (n=5) of the Ca²⁺ current (P<0.05, all, compared with basal; Figure 4).

**Discussion**

In the present study we provide novel evidence for the colocalization of P-type, Q-type, and L-type voltage-activated Ca²⁺ channels in VSMCs obtained from several sources (renal preglomerular resistance vessels and aorta) and from several species (rat and mouse). We used an RT-PCR–based strategy to show that mRNA for both CaV2.1a (P-type) and CaV2.1b (Q-type) splice variants are present in renal smooth muscle cells. Consistent with this, we demonstrated in an aortic smooth muscle cell line that there was an additive reduction of currents through CaV with the L-type–specific blocker calcisepine and the P/Q-type–specific blocker ω-agatoxin IVA. When using low concentrations (10 nmol/L) of ω-agatoxin IVA combined with calcisepine, a residual current remained. This Ca²⁺ current could be nearly abolished by raising the ω-agatoxin IVA concentration of the blocker mixture to 300 nmol/L, a concentration that also blocks Q-type currents. Furthermore, also in freshly isolated mouse renal smooth muscle cells, all 3 types of Ca²⁺ currents were found. Thus, our molecular and functional data demonstrate expression of P- and Q-type channels, as well as L-type channels in rat and mouse VSMCs. Also, antibodies against both CaV1.2 and CaV2.1 label all of the smooth muscle cells present in rat renal preglomerular vasculature, implying that these proteins are localized in the same cells.

The integrated role of several different CaV subtypes in the same smooth muscle cell remains to be resolved. We have observed previously that both L- and P/Q-type Ca²⁺ channels contribute to the depolarization-induced global rise of intracellular Ca²⁺ concentration in renal arterioles.⁴,⁵ These results suggest that the Ca²⁺ currents found in the present article contribute to excitation contraction coupling in intact vessels. In neurons, distinct roles of different CaV subtypes in synapses are established. Thus, in the neuromuscular junctions of mouse urinary bladder, the release of acetylcholine is coupled primarily to N-type channels, whereas ATP release depends on P/Q-type channel activation (reviewed by Waterman¹⁰). The enteric inhibitory motor neurons in the mouse colon release NO and ATP. NO release is governed primarily by Ca²⁺-sensitive NO production, whereas ATP is released through Ca²⁺-sensitive vesicular exocytosis; these processes show a differential dependence on CaV subtypes.¹⁰ It is possible that a similar division of tasks between CaV subtypes exists in smooth muscle cells. The channels could be located in spatially separated areas of the cell membrane and/or coupled to separate intracellular targets, with a need for the activation of channels throughout the membrane to obtain the global rise in [Ca²⁺]i needed for contraction; alternatively, generation of a global rise in [Ca²⁺]i sufficient to induce force generation could require activation of a larger number of channels than is represented by just 1 channel type. In any event, the role of the CaV subtype variation is likely to be found primarily on the level of channel regulation. As to their functional role in excitation–contraction coupling, the distinguishing features of P/Q-type Ca²⁺ currents compared with L-type Ca²⁺ currents are their very slow and Ca²⁺-independent rate of inactivation, with Q-type channels having a slower inactivation rate than P-type channels, and the strong inhibitory modulation by G proteins. The latter feature is involved in hormonal regulation of channel properties.¹¹,¹² Channel regulation by Ca²⁺ binding and phosphorylation also differs between L- and P/Q-type channels. Thus, the presence of P/Q-type currents in the cells could significantly expand the
time for active Ca$^{2+}$ influx and allow hormonal influence on vascular reactivity.

The sensitivity to calcineurin in the present study is higher than reported in some studies (eg, Reference 13). On the other hand, 20 nmol/L calcineurin inhibited a depolarization-induced increase in Ca$^{2+}$ in glomerular arterioles, and Ca$^{2+}$ currents and depolarization-conducted contractions in smooth muscle preparations have been reported to be inhibited with IC$_{50}$ values in the range of 5 to 75 nmol/L.14,15

Immunolabeling of A7r5 cells, freshly isolated renal VSMCs, and renal cryosections showed labeling of all of the preglomerular VSMCs regardless of whether anti-Ca$_{v}$1.2 or anti-Ca$_{v}$2.1 antibody was used, indicating that both channels were expressed in all of the cells. Similarly, our electrophysiological data showed Ca$^{2+}$ currents in all of the renal smooth muscle cells. This result is at variance with the results of Gordienko et al, who reported that only 30% of isolated renal VSMCs contained L-type currents as measured by single-cell patch clamp. The reason for the discrepancy is not clear, but it could be of technical origin, because L-type Ca currents are vulnerable. As reported previously in rat renal myocytes, we found an exquisitely large Ca-current density also in the mouse preglomerular myocytes (6.03±0.60 pA/pF) compared with rat tail artery myocytes (1.4 to 1.8 pA/pF) and rat A7r5 cells (1.3±0.2 pA/pF).

Confocal imaging performed on immunofluorescence-labeled single, freshly isolated renal VSMCs revealed that, although Ca$_{v}$1.2 staining was clearly strongest in the area of the plasma membrane, Ca$_{v}$2.1 staining was found diffusely throughout the cytoplasm. One explanation for this diffuse appearance of Ca$_{v}$2.1 labeling in the cytoplasm could be binding of the antibody to an unspcific, cytoplasmic epitope. However, lines of evidence counter this explanation. First, a BLAST search did not give any hits apart from rat and mouse Ca$_{v}$2.1, indicating that no other known proteins contain epitopes identical to the one recognized by the anti-Ca$_{v}$2.1 antibody. Second, only a single band of the expected size appears in Western blotting on aorta, A7r5 cells, and VSMCs. This would speak against the presence of unspecific epitopes in these cells. Third, in immunohistochemical labelings, Ca$_{v}$2.1 immunoreactivity was restricted to vascular and glomerular structures. Thus, the strong labeling of the cytoplasm of smooth muscle cells with Ca$_{v}$2.1 antibody suggests localization of Ca$_{v}$2.1 protein. Strong expression of a transmembrane protein, such as Ca$_{v}$2.1 in the cytoplasm, could have several explanations. The patch/clamp data indicate that >60% of the Ca$^{2+}$ current in A7r5 cells is because of P- and Q-type channels; thus, this could be associated with a higher level of Ca$_{v}$2.1 than Ca$_{v}$1.2 production and a higher level of immunoreactive Ca$_{v}$2.1 protein being processed within the cell. In addition, the cells could hold intracellular stores of channels, ready to be translocated to the membrane, or Ca$_{v}$2.1 could be involved in signaling in intracellular membranes. In any case, the observation of significant intracellular localization of Ca$_{v}$2.1 warrants additional study. In conclusion, vascular myocytes coexpress L-type and both splice variants of the P/Q-type Ca$_{v}$.s.

**Perspectives**

Dihydropyridine Ca$^{2+}$ channel blockers differ in their selectivity toward L- and P/Q-type Ca$^{2+}$ channels. Thus, amlodipine and cilnidipine inhibit both L- and P/Q-type Ca$^{2+}$ channels, whereas nifedipine and nitrendipine are selective toward L-type channels. Consistent with this, amlodipine reduces renal vascular resistance in spontaneously hypertensive rats, whereas nifedipine has no effect. In humans, amlodipine is associated with glomerular hyperfiltration and adverse effects on albuminuria, whereas nitrendipine has been associated with a reduction in proteinuria. Channel selectivity may, therefore, be a relevant parameter to consider when comparing effects of various Ca$^{2+}$ channel blockers.

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