Mural propagation of descending vasa recta responses to mechanical stimulation

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Zhong Z, Payne K, Cao C, Pallone TL. Mural propagation of descending vasa recta responses to mechanical stimulation. Am J Physiol Renal Physiol 305: F286–F294, 2013. First published May 22, 2013; doi:10.1152/ajprenal.00220.2013.—To investigate the responses of descending vasa recta (DVR) to deformation of the abluminal surface, we devised an automated method that controls duration and frequency of stimulation by utilizing a stream of buffer from a micropipette. During stimulation at one end of the vessel, fluorescent responses from fluo4 or bis[1,3-dibutylbarbituric acid-(5)] trimethineoxonol [DiBAC4(3)], indicating cytoplasmic calcium ([Ca2+]c) or membrane potential, respectively, were recorded from distant cells. Alternately, membrane potential was recorded from DVR pericytes by nystatin whole cell patch-clamp. Mechanical stimulation elicited reversible [Ca2+]c responses that increased with frequency. Individual pericyte responses along the vessel were initiated within a fraction of a second of one another. Those responses were inhibited by gap junction blockade, removal of extracellular Ca2+, inhibition of L-type voltage-gated Ca2+ channels (CaV) with nifedipine, or kinase blockade with 2-morpholin-4-yl-8-phenylglycyrrhetinic acid (100 μM) or phosphoinositide 3 kinase inhibition with 2-morpholin-4-yl-8-phenylchromen-4-one (50 μM). [Ca2+]c responses were blocked by removal of extracellular Ca2+ or L-type voltage-gated channel blockade with nifedipine (10 μM). At concentrations selective for the T-type channel blockade, mibebradil (100 nM) was ineffective. During mechanostimulation, pericytes rapidly depolarized, as documented with either DiBAC4(3) fluorescence or patch-clamp recording. Single stimuli yielded depolarizations of 22.5 ± 2.2 mV while repetitive stimuli at 0.1 Hz depolarized pericytes by 44.2 ± 4.0 mV. We conclude that DVR are mechanosensitive and that rapid transmission of signals along the vessel axis requires participation of gap junctions, L-type Ca2+ channels, and pericyte depolarization.

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Address for reprint requests and other correspondence: T. L. Pallone, Div. of Nephrology, N3W143, 22 S. Greene St., UMMS, Baltimore, MD 21201 (e-mail: tpallone@medicine.umaryland.edu).
one end of the vessel. Motion artifacts occurred only adjacent to the stimulation pipette. Elsewhere, visible cell motion or individual cell contractions were not observed. Away from the stimulation pipette tip, stable measurements are possible by either fluorescence imaging or patch-clamp recording. In vessels that are immobilized without luminal microperfusion, quantifiable wall motions are not observable even when contractile agonists are applied. This permits quantification of fluorescence in regions of interest. Moreover, long-term stable gigaseals needed for electrophysiological recording during mechanical stimulation herein, or in the presence of contractile agonists, are possible (24, 25, 45).

**Fluorescence microscopy.** As previously described, we recorded propagated \([\text{Ca}^{2+}]_{\text{CYT}}\) transients along the DVR wall with the \([\text{Ca}^{2+}]_{\text{CYT}}\) sensitive fluorescent probe, fluo4 (45). As a single wavelength probe with bright emissions, fluo4 enabled image acquisition to be performed at a rapid rate. Fluo4 (Molecular Probes) was loaded by incubation of the AM ester (2 \(\mu\)mol/l, 20 min, 37°C) and excited at 485 nm (DeltaRam, PTI). Fluorescent emissions were monitored at 530 nm (B-2E/C filter cube, Nikon) using a Nikon 60 1.45 N.A. plan apo oil immersion objective. Using fluo4 it was possible to capture sequential images at 300-ms intervals with a low-light CCD camera with on-chip multiplication gain (Photometrics Cascade 512B,
Roper Scientific) using ImageMaster software (PTI). The $[Ca^{2+}]_{CYT}$ transients were quantified off-line from the images by specifying regions of interest (ROI) in NIH ImageJ. In some experiments, pericyte membrane potential was recorded using the voltage-sensitive fluorescent probe bis[1,3-dibutylbarbituric acid-(5)] trimethineoxonol (DiBAC$_4$(3); 5 μM), using methods we previously described (47). DiBAC$_4$(3) was excited at 485 nm, using a DeltaRAM illuminator (PTI). Fluorescent emission at 530 nm was isolated with a band pass filter (Omega Optical, Brattleboro, VT).

Whole cell patch-clamp recording. Patch pipettes were made from borosilicate glass (PG52154-4, external diameter 1.5 mm, internal diameter 1.0 mm; World Precision Instruments, Sarasota, FL), using a two-stage vertical pipette puller (Narishige PP-830) and heat polished. Membrane potential was measured using the nystatin-perforated patch configuration by zero current clamp recording (19) as previously described (25, 50). For patch-clamp, the electrode solution was (in mmol/l) 120 K aspirate, 20 KCl, 10 NaCl, 10 HEPES, pH 7.2, and nystatin (100 μg/ml with 0.1% DMSO) in ultrapure water. Recordings were obtained with a CV201AU headstage and Axopatch 200 amplifier (Molecular Devices, Foster City, CA). Patch-clamp pipettes used for mechanostimulation (Fig. 1) were filled with PSS.

Reagents. Fluo-4 and DiBAC$_4$(3) were from Molecular Probes. 18 β-Glycyrrhetinic acid (18βGRA), 2-morpholin-4-yl-8-phenylchromen-4-one (LY294002) nystatin, collagenase 1A, protease XIV, and other chemicals were from Sigma (St. Louis, MO). Liberase Blenzyne 1 was from Roche Applied Science. 18βGRA was dissolved in DMSO. Reagents were thawed and diluted on the day of the experiment and excess was discarded daily. Blendzyme was stored in 40-μl aliquots of 4.5 mg/ml in water and diluted into high-glucose DMEM lacking serum on the day of the experiment.

Statistics. Data in the text and figures are reported as means ± SE. The significance of differences was evaluated with SigmaStat 3.11 (Systat Software, Point Richmond, CA) using parametric or nonparametric tests as appropriate for the data. Comparisons between two groups were performed with Student’s t-test (paired or unpaired, as appropriate) or the Rank Sum Test (nonparametric). Comparisons between multiple groups were performed with repeated-measures ANOVA, or repeated-measures ANOVA on ranks (nonparametric). Post hoc comparisons were performed using Tukey’s test. $P < 0.05$ was used to reject the null hypothesis.

RESULTS

$DVR [Ca^{2+}]_{CYT}$ responses occur in pericytes. Abuminal mechanostimulation led to elevations of $[Ca^{2+}]_{CYT}$ along the vessel axis. Figure 1 illustrates the method and typical responses. Figure 1A shows a white-light image of a DVR on a coverslip with abuminal pericyte cell bodies labeled 1–4. A corresponding fluorescent image is provided as Fig. 1B. The fluo-4 $[Ca^{2+}]_{CYT}$ response of each cell is displayed in Fig. 1C as fluo-4 fluorescence normalized to that quantified from the first image (Fo). During stimulation at 0.1 Hz (duration 1 s, every 10 s), simultaneous elevations of $[Ca^{2+}]_{CYT}$ occur in the four pericytes. The region of the abscissa defined by the horizontal dark line at the onset of stimulation in Fig. 1C is expanded as Fig. 1D to show that individual pericyte $[Ca^{2+}]_{CYT}$ responses begin within a fraction of a second of one another (Fig. 1D, *).

As shown in Fig. 2, when the frequency of stimulation was increased from 0.1 to 0.5 Hz, the $[Ca^{2+}]_{CYT}$ response was sustained rather than oscillatory. Figure 2A shows normalized fluo-4 fluorescence from pericytes, analyzed by placing ROI over abuminal protrusions of the pericyte cell bodies. The means ± SE of pericyte responses are shown at baseline and during subsequent applications of 0.1- and 0.5-Hz stimulations. At 0.1 Hz, the pericyte $[Ca^{2+}]_{CYT}$ responses are rapidly reversible, nearly returning to baseline. In contrast, more rapid 0.5-Hz stimulation (1-s duration separated by 1-s interval) leads to larger and more persistent $[Ca^{2+}]_{CYT}$ elevations. Endothelial effects are shown in Fig. 2B where fluorescence has been quantified by placing ROI over adjacent endothelial cell bodies. The image analysis exemplified in Fig. 2C where “p” and “e” represent a pericyte and an endothelial cell, respectively. We cannot rule out that the small $[Ca^{2+}]_{CYT}$ elevations in Fig. 2B arise from fluo4 fluorescence emanating from pericytes that wrap their extensions around the vessel wall. Nonetheless, it is clear from comparison of the data in Fig. 2, A and B, derived from the same series of vessels, that the predominant $[Ca^{2+}]_{CYT}$ elevations occur in pericytes and not the endothelium. This is illustrated by the succession of images in Fig. 2C where a fluorescence pericyte (p) is nearly invisible at baseline but appears during 0.1- or 0.5-Hz stimulation. Fluorescence of an adjacent endothelial cell (e) is greater at onset, but remains stable. As we previously described, the greater baseline fluorescence of endothelia occurs because they load fluorescent dyes by deesterification more efficiently than the adjacent pericytes (26, 27).
Pericyte \([\text{Ca}^{2+}]_{\text{CYT}}\) responses require gap junctions and kinase activation. Figure 3A illustrates the ability of gap junction blockade with 18\(\beta\)-glycerethinic acid (18\(\beta\)GRA) (100 \(\mu\)M) to prevent transmission of mechanosensitive signaling. Sequential images (300-ms intervals) were captured at 30-s baseline upon which 0.1-Hz stimulation was initiated for 120 s. Subsequently, 18\(\beta\)GRA was exchanged into the bath and the stimulation protocol was reinitiated and repeated again after washout. Statistical comparison of the peak \([\text{Ca}^{2+}]_{\text{CYT}}\) elevations before, during, and after 18\(\beta\)GRA is summarized in Fig. 3B. 18\(\beta\)GRA effectively and reversibly blocked the pericyte \([\text{Ca}^{2+}]_{\text{CYT}}\) responses. Mechanosensitivity of blood vessels has been traced to integrin signaling through phosphoinositide-3-kinase (PI3K) (1, 2). Accordingly, we examined the effects of PI3K blockade with LY294002. As shown in Fig. 4, A and B, using a stimulation protocol identical to that in Fig. 3, LY294002 reversibly blocked pericyte responses.

Pericyte \([\text{Ca}^{2+}]_{\text{CYT}}\) responses require external calcium entry. As illustrated in Fig. 5, removal of \(\text{Ca}^{2+}\) ions from the bath (0 \(\text{CaCl}_2\)/100 \(\mu\)M EGTA) lowered baseline fluo4 fluorescence and prevented any pericyte \([\text{Ca}^{2+}]_{\text{CYT}}\) responses to mechanostimulation from occurring. This supports dependence of responses on \(\text{Ca}^{2+}\) entry. We previously showed that DVR pericyte \(\text{Ca}^{2+}\) entry is partially mediated by nifedipine- and diltiazem-sensitive L-type CaV (45, 48, 50). In this study, we tested whether that pathway also mediates \(\text{Ca}^{2+}\) entry during mechanical stimulation. As shown in Fig. 6A, nifedipine effectively reduced the responses but was slow to reverse during washout. The stimulation protocol used in Figs. 3 and 4 was repeated before, during, and after nifedipine application (Fig. 6B) with results summarized in Fig. 6C. Although T-type CaV have been observed in DVR and participate in efferent arteriolar contractile responses (18, 33), we and others have been unable to detect T-type currents during patch-clamp studies (39, 48). Herein, we tested whether T-type channels participate in \(\text{Ca}^{2+}\) entry during mechanostimulation by examining the effectiveness of a low concentration of mibebradil (Fig. 7A). At a concentration of 100 nM, selective for T-channel blockade, it was ineffective. As illustrated in Fig. 7B, raising mibebradil concentration to 10 \(\mu\)M partially blocked pericyte \([\text{Ca}^{2+}]_{\text{CYT}}\) responses. Those results mirror blockade of angiotensin II-induced contraction of isolated-perfused DVR by high concentrations where L-type channel inhibition is also likely to occur (48).

Mechanostimulation depolarizes the pericyte cell membrane. Since \(\text{Ca}^{2+}\) entry via nifedipine-sensitive L-type channels is gated by membrane depolarization, we tested for pericyte depolarization during mechanostimulation. Using DiBAC_4(3) as a voltage-sensitive probe, baseline fluorescence in the absence of stimulation tended to slowly increase as we previously observed (47). DiBAC_4(3) responds too slowly to resolve the detailed kinetics of voltage fluctuations, but did confirm pericyte depolarization (Fig. 8).
In contrast to DiBAC4(3), electrophysiological measurement can track rapid changes of membrane potential but dialyzes the cell cytoplasm with electrode buffer (19). Despite this, the nystatin-perforated patch-clamp with 100 nA current recording also confirmed that mechanostimulation depolarizes pericytes. As shown in Fig. 9A, solitary stimulations yielded rapid and reversible increases of membrane potential the magnitude of which correlated with the pressure applied to the stimulation pipette. Contrast of the effect of solitary stimulation vs. 0.1-Hz repetitive stimulation is provided in Fig. 9B and summarized in Fig. 9C and D. Single stimulations yielded a 22.5 ± 2.2-mV rise in membrane potential while repetitive stimulation nearly doubled the depolarization to 44.2 ± 4.0 mV.

**DISCUSSION**

DVR are small microvessels (~13-μm internal diameter on average) that derive from juxtamedullary efferent arterioles to supply the medulla of the kidney with blood flow. Luminal pressures are typically 10 to 12 mmHg by servo nulling measurements in the accessible, papillary inner medulla. When DVR are blocked with wax and perfused retrograde from the papilla toward the outer medulla, pressures rise to 20 to 22 mmHg providing a probable upper limit on luminal pressures of DVR that lie in outer medullary vascular bundles (29). Such pressures are well below the range that elicits myogenic contraction of renal intralobar or afferent arterioles. Despite this, DVR have been found to be very sensitive to mechanical stimuli. Luminal pressurization or step changes in rate of microperfusion elicit \[Ca^{2+}\]_{CYT} responses and modify NO generation. Due to the small size of these vessels, it has not been possible to reliably separate luminal shear from wall stretch as the mechanical stimulus (49). As an alternative, we demonstrated that manually puffing a buffer stream from a micropipette onto the abluminal DVR wall elicits \[Ca^{2+}\]_{CYT} responses that propagate along the vessel axis (44). In this study, we automated that method and reduced the stimulation pipette orifice to that typical of a patch-clamp pipette (~1 μm). These alterations improve reproducibility of experimental conditions and remove variations that result from manual control of pipette pressure by the investigator (Fig. 1). The automation enables reliable superposition and averaging of results from individual vessels to enhance power of statistical comparisons (Figs. 2-8). Immobilization of the vessel away from the stimulation pipette prevents motion artifacts and cell contractions are not observed to an observable degree. This is consistent with our prior observation that luminal pressurization with microperfusion is needed to quantify wall motion and vasoactivity in these small vessels. Finally, it is clear that motion artifacts away from the site of stimulation are sufficiently small for gigaohm seals to be maintained for electrophysiological measurements. Similarly, in past studies, we have been readily able to sustain such seals when vasoactive agonists are applied (24, 25, 45).

In addition to automation of stimulus, rapid acquisition, low-light imaging with a 16-bit camera is important to spatially analyze responses. As previously observed with fura2, deesterification of fluo4 during loading yields endothelial fluorescence that exceeds the baseline pericyte signal (Fig. 2C) (26, 45). Pericyte responses can nonetheless be quantified by analyzing cell bodies that jut out from the abluminal surface away from of the vessel wall (Fig. 2A). The 16-bit capabilities of modern CCD cameras (>65,000 gray levels) provide sufficient sensitivity to accurately quantify fluorescence changes in these small cells. Accurate quantification of endothelial signals without pericyte contamination is more problematic because pericyte extensions of submicron thickness wrap around the vessel wall. As a consequence, the small elevations of fluo4 fluorescence obtained by placing ROI over endothelia in Fig. 2B might arise from pericytes. Despite these limitations, comparison of the dramatic differences between Fig. 2A and Fig. 2B favors the interpretation that \[Ca^{2+}\]_{CYT} responses are largely confined to the pericytes.

The present study confirms that physiological responses can be rapidly transmitted along the DVR wall (Fig. 1) by mechanisms that are sensitive to blockade of gap junctions (Fig. 3). With regard to cell coupling within the DVR wall, prior studies have pointed to syncytial continuity of the endothelium. Dialysis of Lucifer yellow (457 Da) into an endothelial cell from a

**Fig. 5. Elimination of pericyte \[Ca^{2+}\]_{CYT} responses by removal of extracellular \[Ca^{2+}\] ions. A: examples show the effect of removing \[Ca^{2+}\] ions from extracellular buffer during (Aa) or before (Ab) 0.1-Hz stimulation. In the experiments, \[Ca^{2+}\] was nominally reduced to 0 concentration in the external buffer and chelated by addition of 100 μM EGTA (total, \(n = 12\) pericytes from 9 DVR). B: statistical comparison of data by comparing the mean of peaks from each vessel in 1 and 0 mM CaCl_{2}/EGTA. Residual peaks were never detectable in 0 \[Ca^{2+}\] so that the fluorescence of the baseline was used for comparison to peaks in 1 mM CaCl_{2} (**\(P < 0.01\), 1 vs. 0 mM CaCl_{2}/EGTA).**
Mibefradil was only effective at the higher nonselective concentration (P < 0.05, mean of peaks in 10 µM mibefradil vs. either 0 or 100 nM mibefradil).

Fig. 6. Blockade of pericyte [Ca2+]cyt responses by nifedipine. A: example shows the mean response of 5 pericytes to introduction and washout of nifedipine (10 µM) during 0.1-Hz stimulation. Nifedipine reversibly suppressed [Ca2+]cyt responses but its effects were slow to reverse. B: means ± SE of normalized fluo4 fluorescence are shown for pericytes stimulated before (black), during (red), and after washout (blue) of nifedipine (10 µM). In each sequence, images were acquired for 30 s before onset of 0.1-Hz stimulation. The bath was exchanged between sequence acquisitions and the data were superimposed for display. Each vessel was analyzed as the mean of its pericyte responses and the means ± SE of individual vessels were displayed from that data (total, n = 25 pericytes from 5 DVR). C: statistical comparison of data from B performed by comparing the mean of peaks from each vessel before, during, and after washout of nifedipine (*P < 0.05 nifedipine vs. control).

Fig. 7. Blockade of pericyte [Ca2+]cyt responses by mibefradil. A: means ± SE of normalized fluo4 fluorescence are shown for pericytes stimulated at 0.1 Hz before, during, and after washout of mibefradil (100 nM, n = 29 pericytes, 8 vessels). At 100 nM aimed at selectively blocking T-type voltage-gated Ca2+ channels, mibefradil was without effect. B: means ± SE of normalized fluo4 fluorescence are shown for pericytes stimulated at 0.1 Hz before and during sequential introduction of mibefradil at 100 nM and 10 µM (n = 25 pericytes, 5 vessels). Mibefradil was only effective at the higher nonselective concentration (P < 0.05, mean of peaks in 10 µM mibefradil vs. either 0 or 100 nM mibefradil).

Fig. 8. Effect of 0.1-Hz stimulation on pericyte membrane potential recorded with bis[1,3-dibutylbarbituric acid-(5)] trimethineoxonol [DiBAC4(3)]. DVR, isolated and adherent to coverslips as in Fig. 1, were loaded with the voltage-sensitive probe DiBAC4(3). Images were captured with and without 0.1-Hz stimulation (n = 9 DVR, 40 pericytes). Fluorescence was normalized to that in the first image (F/Fo). Each vessel was analyzed as the mean of its pericyte responses and the means ± SE of individual vessels were displayed from that data (*P < 0.05, no stimulation vs. 0.1-Hz stimulation for all time points beyond 30 s).
gap junction blockade (Fig. 3), participation of the syncytial endothelial layer in voltage spreading that yields simultaneous depolarizations of pericytes seems very likely.

While the existence of mechanically stimulated DVR pericyte depolarization is clear (Figs. 8 and 9), the channel architecture and ion movements that underlie the response are uncertain. Those events might originate in pericytes or they might arise from endothelia through syncytial spread of monovalent charge to pericytes. The coincidence of pericyte responses in Fig. 1C shows that cells separated by distances of 100 μm depolarize within milliseconds of one another, likely too fast to invoke simple diffusion of a vasoactive mediator; it occurs with a speed reminiscent of neural transmission. Depolarization of isolated vascular smooth muscle generally involves cation entry, reduction of potassium conductance, an increase in chloride channel activity, or a combination thereof and we showed that such channel modulation occurs in response to angiotensin II stimulation of DVR pericytes (24, 25, 50). With regard to enhancement of cation conductance, activation of transient receptor potential channels by mechanical stretch has been implicated (12, 43). Increasing attention has been paid to plausible roles of epithelial Na⁺/H⁺ channel/degenerin proteins (9, 10), although detection of epithelial Na⁺ channels currents in vascular smooth muscle has been lacking (8, 22, 40). Other possibilities exist. Direct control of L-type channels by integrin interactions (13, 42) or through PI3K (1,
analyze the responsible ion movements and channel activity. During mechanostimulation may provide means to eventually discern the ability to perform patch-clamp studies during mechanostimulation may provide means to eventually analyze the responsible ion movements and channel activity.

Prior studies of Ca\(^{2+}\) entry routes into DVR pericytes identified an important role for Ca\(^{2+}\) in agonist-invoked contractions. Predominance of Ca\(^{2+}\) as a route for Ca\(^{2+}\) entry varies along the renal vasculature and may not be invoked in a given segment by all contractile agonists (16, 17, 32). In our hands, electrophysiological studies of pericytes showed that voltage clamp depolarization gates sequential inward currents carried by tetrodotoxin-sensitive Na\(^{+}\) channels and nifedipine-sensitive L-type Ca\(^{2+}\) channels (20, 46, 48). We found that L-type blockers, nifedipine and diltiazem, interfere with agonist-induced vasoconstriction by angiotensin II and that oscillatory inward currents involving Cl\(^{-}\) ions can be involved (48, 50). A role for L-type Ca\(^{2+}\) in the mechanosensitive responses of this study is supported by the ability of nifedipine to block [Ca\(^{2+}\)]\(_{\text{cyt}}\) transients (Figs. 6 and 7). Concomitant depolarization of the cell membrane (Figs. 8 and 9) points to processes intended to gate Ca\(^{2+}\) channels, but the means used to achieve pericyte depolarization may be quite different from that involved in agonist contractions.

The vasculature of the kidney and renal medulla is subject to various mechanical stimuli. Classically, endothelial shear forcing vasodilator formation and wall stretch favoring myogenic constriction have received greatest attention. Whether the method of stimulation we describe in Fig. 1 evokes responses related to myogenic mechanisms is highly uncertain. As described above, DVR exist in the low-pressure efferent juxtedudillary region of the renal circulation. We have been unable to detect overt myogenic contraction of DVR in response to raising luminal pressure (49); however, recent studies in human DVR by Sendeski and colleagues (37) identified such behavior. In addition to pressure-associated variations of luminal flow and pressure, ureteral peristalsis regularly compresses the papillary inner medulla to optimize urinary concentration (36). The mechanism by which such peristalsis modifies medullary gradients has been the subject of past research and much controversy. If DVR respond to mechanical compression from ureteral peristalsis, feedback mechanisms may exist to optimize medullary perfusion for urinary concentration as part of the process. Finally, the renal medulla is believed to have sparse innervation. The speed with which responses spread along the vessel axis (Fig. 1) raises the possibility that neural control in the deep cortex is relayed to DVR of the outer medulla through the endothelial syncytium rather than renal nerves, per se.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: Z.Z., C.C., and T.L.P. conception and design of research; Z.Z. and K.P. performed experiments; Z.Z., K.P., T.L.P. analyzed data; Z.Z. and T.L.P. prepared figures; Z.Z., K.P., C.C., and T.L.P. approved final version of manuscript; C.C. and T.L.P. interpreted results of experiments; T.L.P. drafted manuscript; T.L.P. edited and revised manuscript.

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