Descending vasa recta endothelial cells and pericytes form mural syncytia

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Zhang Z, Lin H, Cao C, Payne K, Pallone TL. Descending vasa recta endothelial cells and pericytes form mural syncytia. Am J Physiol Renal Physiol 306: F751–F763, 2014. First published December 31, 2013; doi:10.1152/ajprenal.00470.2013.—Using patch clamp, we induced depolarization of descending vasa recta (DVR) pericytes or endothelia and tested whether it was conducted to distant cells. Membrane potential was measured with the fluorescent voltage dye di-8-ANEPPS or with a second patch-clamp electrode. Depolarization of an endothelial cell induced responses in other endothelia within a millisecond and was slowed by gap junction blockade with heptanol. Endothelial response to pericyte depolarization was poor, implying high-resistance myo-endothelial coupling. In contrast, dual patch clamp of neighboring pericytes revealed syncytial coupling. At high sampling rate, the spread of depolarization between pericytes and endothelia occurred in 9 ± 2 or 12 ± 2 μs, respectively. Heptanol (2 mM) increased the overall input resistance of the pericyte layer to current flow and prevented transmission of depolarization between neighboring cells. The fluorescent tracer Lucifer yellow (LY), when introduced through ruptured patches, spread between neighboring endothelia in 1 to 7 s, depending on location of the flanking cell. LY diffused to endothelial cells on the ipsilateral but not contralateral side of the DVR wall and minimally between pericytes. We conclude that both DVR pericytes and endothelia are part of individual syncytia. The rate of conduction of membrane potential exceeds that for diffusion of hydrophilic molecules by orders of magnitude. Gap junction coupling of adjacent endothelial cells may be spatially oriented to favor longitudinal transmission along the DVR axis.

Endothelia and smooth muscle of blood vessels communicate through connexon channels that facilitate cell-to-cell signal transmission. The gap junctions they form tend to be prominent in small resistance vessels. The roles of gap junctions have been frequently reviewed (2, 11, 17, 33, 49). Endothelial responses to vasodilators often lead to membrane hyperpolarization that is conducted along the vessel wall (2). Similarly, depolarizing contractile responses may be conducted between smooth muscle cells (19). Rapid electrical transmission through gap junctions may expand the range of paracrine vasodilators to overcome upstream resistance that otherwise limits increases of nutrient blood flow. The effects of endothelial hyperpolarization may also provide a brake on vasoconstriction by transmitting signals to vascular smooth muscle across myo-endothelial gap junctions. Descending vasa recta (DVR) are 10- to 15-μm microvessels that distribute blood flow to the outer and inner medulla of the kidney (38, 43). The DVR wall is comprised of a layer of endothelial cells surrounded by smooth muscle-like pericytes. Connexins 37, 40, 43, and 45 are expressed by the DVR wall (20, 54). Conducted vasodilatory responses may be important to medullary function because sufficient perfusion is needed to avoid hypoxia and acute renal injury (23). Conversely, conducted contractile responses may ensure against excess perfusion that might interfere with efficient countercurrent exchange thereby reducing corticomedullary solute gradients and optimal urinary concentration (40).

The DVR endothelium is a highly coupled electrical syncytium (54). The extent of cell-to-cell coupling within the pericyte layer has been less certain. Electrical spreading of charge from the DVR wall can be observed in explanted vessels. When the membrane potential of a coupled cell is abruptly changed by voltage clamp, prolonged “capacitance transients” are observed in the current records due to spreading of charge to adjacent cell membranes. Long transients are always observed when explanted DVR endothelium is patch clamped, but only variably found in pericytes (54). When the fluorescent tracer Lucifer yellow (LY; molecular mass 457 Da) is introduced into the cytoplasm from a patch pipette, it diffuses freely between adjacent endothelial cells but is more confined in pericytes (54). Based on those observations, we surmised that endothelia are connected as a syncytium while pericyte coupling may be variable or of high resistance. In a recent study, we obtained additional evidence that favors pericyte coupling. Mechanical deformation of a pericyte surface induced a propagated cytoplasmic calcium ([Ca2+]cyt) response. Transmission to other pericytes occurred within milliseconds and involved depolarization, voltage-gated Ca2+ channel activation, and gap junction communication. Parallel changes within the underlying endothelium were not found (61).

In the present study, we tested whether pericytes are indeed syncytial, and whether the velocity of cell-to-cell conduction might be explained by transfer of a diffusible molecule. In one series, we loaded the fast-response voltage dye di-8-ANEPPS into DVR and measured its fluorescence while either an endothelial cell or pericyte was depolarized by voltage clamp from a distant electrode. In a parallel effort, dual cell patch clamp was used so that membrane potential deviations in nearby cells could be directly measured when a patched cell was depolarized by voltage clamp or current injection. We find that membrane potential perturbations can be transmitted along either the pericyte or endothelial layers on a time scale of microseconds. Using fluorescence videomicroscopy, we extended past studies with LY by measuring its rate of diffusion. LY diffused between adjacent endothelial cells on a time scale of several seconds but failed to spread to the contralateral side of the vessel. We conclude that both the pericyte and endothelial layers are syncytia, connections between pericytes are of higher resistance than those between endothelia, and that endothelial gap junctions may be spatially oriented to favor axial over lateral conduction of electrical signals and diffusible molecules.
METHODS

Isolation of DVR. Investigations involving animal use were performed according to protocols approved by the Institutional Animal Care and Use Committee of the University of Maryland. Kidneys were harvested from Sprague-Dawley rats (120–200 g; Harlan) that had been anesthetized by an intraperitoneal injection of ketamine/xylazine (80 mg/kg/10 mg/kg). Tissue slices were stored at 4°C in a physiological saline solution (PSS) containing (in mM) 145 NaCl, 5 KCl, 1 MgCl2, 1 CaCl2, 10 HEPES, 10 glucose, pH 7.4 at room temperature. As previously described, DVR were explanted by hand dissection. The vessels were largely derived from vascular bundles of the inner stripe of the outer medulla (35, 41). Once isolated, they were transferred to an inverted microscope for fluorescence microscopy and patch-clamp studies. Isolated vessels are typically 500 to 1,000 μm long. One rat was killed per day, generally in the late morning, and DVR were harvested as needed through mid to late afternoon. Access to endothelial cells for patch clamp necessitated stripping of pericytes from the abluminal surface. This was done, as previously described and illustrated in detail, by drawing and then expelling a collagenase-treated vessel into the mouth of the micropipette the orifice of which was heat polished to ~6 μm (48, 54). For protocols that involve pericyte removal, tissue digestion was performed at 37°C for 22 min in a cocktail of collagenase 1A (0.5 mg/ml), protease XIV (0.4 mg/ml), and bovine serum albumin (1.0 mg/ml) in PSS (in mM: 155 NaCl, 5 KCl, 1 MgCl2, 1 CaCl2, 10 HEPES, and 10 glucose, pH 7.4). For other patch-clamp studies, enzymatic digestion was performed with Blendzyme 1 (0.27 mg/ml, Roche) in high-glucose DMEM media without serum (Invitrogen) at 37°C for 30 min. In all cases, after digestion, tissue was stored in PSS on ice (~4°C) until DVR isolation.

Whole cell patch-clamp recording. Patch pipettes were made from borosilicate glass (PG52151–4, 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 specified levels while di-8-ANEPPS fluorescence was measured using a photon-counting photomultiplier (PMT) assembly (D104B, Photon Technology International). The PMT window was separated from the pipette by ~100–200 μm to avoid artifacts related to shadowing and reflections from the pipette. The rectangular PMT window was adjusted to ~50 μm of vessel length so that the signal was derived from several cells. The window width was adjusted to slightly exceed the vessel diameter. Background fluorescence was determined for each experiment by moving the vessel out of the window at the end of the experiment. The di-8-ANEPPS membrane potential probe was excited at 485 nm, using a DeltaRAM illuminator (PTI) and its emissions were detected at a 530-nm band pass filter (Omega Optical, Brattleboro, VT). In one series, elevation of extracellular KCl concentration (by isomotic substitution for NaCl) was used in place of voltage clamp to depolarize the cells.

Fluorescence videomicroscopy of LY. In some studies, LY was included in the electrode and allowed to diffuse into the cytoplasm of either an endothelial cell (stripped vessel) or pericyte (intact vessel) through a ruptured patch (54). Capacitance transients were monitored by conventional methods as negative pressure was applied to the electrode to rupture the patch. Just before rupture, image capture was initiated using a low-light CCD camera with on-chip multiplication gain (Photometrics Cascade 512B, Roper Scientific) and ImageMaster software (PTI). Sequential images were acquired every 30 ms for 2 min (2,000 images). As previously described, images from the video files were analyzed by region-of-interest analysis using NIH Image J software (61).

Reagents. LY and di-8-ANEPPS were from Invitrogen. Nystatin, heptanol, collagenase 1A, protease XIV, and other chemicals were from Sigma (St. Louis, MO). Heptanol was used throughout at a concentration of 2 mM. Liberase Blenzyme 1 was from Roche Applied Science. 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 based on the number of vessels. Experiments in which gigaseals failed before or during data acquisition were discarded. The significance of differences was evaluated with SigmaStat 3.11 (Systat Software, Point Richmond, CA) using parametric or nonparametric tests or ANOVA as appropriate for the data.

RESULTS

Verification of di-8-ANEPPS using KCl depolarization. Fluorescence of di-8-ANEPPS rises as membrane polarization declines (31, 45). In a first series, we verified the ability of di-8-ANEPPS to track endothelial membrane potential during elevation of extracellular KCl concentration. KCl was sequentially raised while fluorescence was measured by photomultiplier (PMT; Fig. 1A). Means ± SE of di-8-ANEPPS fluorescence, normalized to its initial value (F/Fo), is shown in Fig. 1B as KCl was increased from 5 to 10, 15, 20, 50, and finally 100 mM. At higher concentrations, di-8-ANEPPS fluorescence increased, indicating depolarization. At the lower concentrations of 10 and 15 mM, fluorescence declined indicating hyperpolarization (summarized in Fig. 1C). The ability of small elevations of external KCl to activate strong inward rectifier K+ channels and slightly hyperpolarize the endothelium has been previously demonstrated. Thus, di-8-ANEPPS is sufficiently sensitive to quantify that small effect (5, 6, 61).

ANEPPS (12 μM) was loaded into the DVR for 30 min at room temperature. Either an endothelial cell (in a pericyte-striped vessel) or a pericyte (intact vessel) was concomitantly subjected to whole cell patch clamp and held at ~90 mV. The patched cell was depolarized to specified levels while di-8-ANEPPS fluorescence was measured with a photon-counting photomultiplier (PMT) assembly (D104B, Photon Technology International). The PMT window was separated from the pipette by ~100–200 μm to avoid artifacts related to shadowing and reflections from the pipette. The rectangular PMT window was adjusted to ~50 μm of vessel length so that the signal was derived from several cells. The window width was adjusted to slightly exceed the vessel diameter. Background fluorescence was determined for each experiment by moving the vessel out of the window at the end of the experiment. The di-8-ANEPPS membrane potential probe was excited at 485 nm, using a DeltaRAM illuminator (PTI) and its emissions were detected at a 530-nm band pass filter (Omega Optical, Brattleboro, VT). In one series, elevation of extracellular KCl concentration (by isomotic substitution for NaCl) was used in place of voltage clamp to depolarize the cells.

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Verification of di-8-ANEPPS using voltage-clamp depolarization. We accessed the DVR endothelial cytoplasm by nystatin-perforated patches and tested the ability of di-8-ANEPPS to track voltage clamp-induced depolarization. The patched cell was held at $-90\,\text{mV}$ and sequentially depolarized while di-8-ANEPPS fluorescence was quantified (Fig. 2A). As shown in Fig. 2, B and C, the probe tracked the voltage of the patch pipette and the response was linear (7%/100 mV change in voltage clamp). The data in Fig. 2B display di-8-ANEPPS fluorescence normalized to its value at $-90\,\text{mV}$ (F/Fo). The ordinate in Fig. 2C shows the change in fluorescence expressed as F/Fo. Note that the true calibration factor for di-8-ANEPPS is greater than 7%/100 mV because a voltage drop occurs between the voltage-clamped cell and the cells in the PMT window. This occurs because current leaks to the bath (ground) via cell membranes and a voltage drop occurs between adjacent cells across their gap junctions. In dual patch-clamp experiments, we found a 16% decline so that a more accurate calibration factor for di-8-ANEPPS in this preparation is 8.4%/100 mV.

Tracking of depolarizing pulses by di-8-ANEPPS. To study responses of di-8-ANEPPS, the same configuration as in Fig. 2A was used. The voltage-clamp electrode was held at $-90\,\text{mV}$ and depolarized to $-10\,\text{mV}$ for either 100 or 1,000 ms (Fig. 3). Results from 3 sequential 100-ms pulses are summarized in Fig. 3A. The rise in membrane potential was rapid and reversible but only achieved $\sim 15\,\text{mV}$ depolarization over the 100-ms interval. In contrast, over 1,000 ms, the change in membrane potential more closely approached a full 80-mV change induced by the patch electrode (Fig. 3B). The data in Fig. 3, A and B, were converted to estimate membrane potential (mV) using the 8.4% change per 100 mV, as described above. Fits of the individual data to a single exponential yielded a time constant of $0.61\pm0.12\,\text{s}$ (means $\pm\text{SE}$) reflecting the time required for charge to transfer along the vessel wall and depolarize distant cells.

To examine the rate at which the depolarizing pulse was conducted from the electrode to the adjacent endothelium, the PMT output was recorded at 25 kHz. Two expansions of the data points near the initiation of the depolarizing pulse are provided in Fig. 3C and D. They show that di-8-ANEPPS fluorescence increases within a millisecond of onset of depolarization. Taken together, the data favor the interpretation that the slow rise of fluorescence in Fig. 3, A and B, is not accounted for by an inability of di-8-ANEPPS to respond on the time scale of the experiment. Instead, the need to transfer current through gap junctions to depolarize adjacent cell membranes limits depolarization rate.

To test whether increasing gap junction resistance to charge transfer delays the rate of rise of di-8-ANEPPS fluorescence, the experiments illustrated in Fig. 4 were performed. In one series (Fig. 4A), DVR endothelial cells, stripped of pericytes, were subjected to 100-ms depolarizing pulses ($-90\,\text{to}\,-10\,\text{mV}$) in the presence or absence of the gap junction blocker.
heptanol (2 mM). Heptanol reduced the rate of depolarization of distant cells (Fig. 4C), a finding that is consistent with gap junctions as the dominant resistance to flow of current. Interestingly, in the presence of heptanol, endothelia in the PMT window depolarized for a brief period after the patched cell was repolarized. This can be explained by delayed transfer of charge from partially depolarized cells both forward to the PMT window and in reverse toward the patch electrode via high-resistance, partially blocked gap junctions. In a second series (Fig. 4B), a pericyte was depolarized to determine the ability of charge to transfer from a pericyte to the endothelium. Unlike the rapid rise of di-8-ANEPPS fluorescence that results from depolarization of an endothelial cell, depolarization of a pericyte had minimal effects. These data are consistent with the interpretation that myo-endothelial junctions, if present, are of high resistance.

Dual patch clamp of endothelia and pericytes. The data shown in Fig. 3, C and D, indicate that the time delay between onset of the depolarizing pulse in one endothelial cell and its detection by di-8-ANEPPS in neighboring cells is <1 ms. To corroborate this and more accurately resolve the cell-to-cell transmission rate, we performed dual electrode patch clamp using the configurations illustrated in Fig. 5, A and B. In each case, one patch pipette was used to clamp voltage and depolarize “CellI” while a second electrode, held in zero current-clamp mode, monitored membrane potential at “CellII.” CellII was held at −80 mV and depolarized to −40 mV. To detect the delay from CellI to CellII, we sampled at the maximum possible rate of the analog-to-digital converter (250 kHz) and limited acquisition time to 15 ms to prevent unwieldy data files.

As expected from axial voltage drop between patch site and adjacent cells, in both dual pericyte and dual endothelial recordings (Fig. 5C), prepulse membrane potential at CellII was higher than the holding potential at CellI (−80 mV). In the case of endothelia, the decline between CellI and CellII was 16% of the holding potential and that value was combined with the slope of the response in Fig. 2C to approximate the di-8-ANEPPS calibration factor of 8.4% per 100 mV used in Fig. 4. In both cell types, depolarization of CellII led to a rapid rise in membrane potential at CellII favoring the interpretation that both layers are syncytia. The rate of depolarization at CellII was more rapid for endothelia than pericytes, suggesting that coupling between endothelia is of lower resistance than that between pericytes. Fit of single exponentials to individual responses yielded time constants of 10 ± 2.9 and 34 ± 15 ms, for endothelial and pericyte depolarization rates, respectively (P < 0.01). Due to the high sampling rate (250 kHz, 1 sample every 4 µs), we could resolve a delay between CellI and arrival of depolarization at CellII. Figure 5D shows a single
example of endothelial-to-endothelial transmission where the delay was \( \sim 16 \mu s \). All recordings were similar. Experiments in pericytes and endothelia showed average delays of \( 12 \pm 2 \) and \( 9.4 \pm 2 \mu s \), respectively (N.S.). Based on an intercellular distance of \( \sim 100 \mu m \), conduction velocity must exceed 10 m/s.

**Syncytial coupling of DVR pericytes.** We previously showed that transmission of pericyte \([\text{Ca}^{2+}]_{\text{cyt}}\) responses along the vessel axis can be eliminated by gap junction blockade (61). To corroborate syncytial coupling, we performed additional experiments. In a first series, the experiment illustrated in Fig. 5B was repeated at a slower sampling rate with a longer period of observation. We also used ruptured rather than nystatin-perforated patches so that the effects of lack of rupture could be compared with the ability of heptanol to block pericyte-to-pericyte transmission. An example of voltage responses at Cell2 in the presence and absence of heptanol is shown in Fig. 6A. When the patch at Cell1 was not ruptured, Cell2 did not respond and recordings appeared similar to those in heptanol (not shown). Statistical analysis of the prepulse and endpulse membrane potential at Cell2 is shown in Fig. 6B. Inclusion of heptanol or lack of cytoplasmic access prevented either the prepulse holding potential or subsequent depolarization from affecting Cell2.

We used the “membrane test” feature of pClamp (Molecular Devices) to calculate overall input resistance of the pericyte layer. The software calculates the time constants, access resistance, and membrane resistance from the current records generated during square wave depolarizations (+5 mV) from a holding potential (−80 mV) thus evaluating the preparation as though it is a single large cell. The calculated input resistance in heptanol was more than 10-fold higher than that in vehicle (Fig. 7). This analysis illustrates the ability of heptanol to isolate the patch cell from its neighbors. It does not rigorously simulate the equivalent circuit of a gap junction-coupled prep-
Fig. 4. Effect of endothelial vs. pericyte depolarization on di-8-ANEPPS fluorescence. A and B: schematic depiction of DVR endothelia (dark blue) and abluminal pericytes (black) interconnected by gap junctions (red) and loaded with di-8-ANEPPS. Configurations shown in A and B were used to record fluorescence during depolarization of either a voltage-clamped endothelial cell (stripped vessel) or pericyte (intact vessel) of the DVR wall. C: endothelia were held at −90 mV and depolarized to −10 mV for 100 ms in the presence or absence of heptanol (2 mM, n = 5 vessels, 3 rats). Heptanol markedly delayed and diminished the rise in fluorescence. When a pericyte was depolarized by voltage clamp (n = 7, 4 rats), the rise in di-8-ANEPPS fluorescence was minimal, implying poor transmission of membrane potential to the underlying endothelial layer.

aration wherein serial resistances separate successive cell capacitances along the vessel axis.

In a final series, we used current-clamp protocols to test the ability of heptanol to affect the membrane potential of Cell2 when depolarizing currents were injected into Cell1 (Fig. 8). Cell1 was held at a current of −70 pA and subjected to injections of current (abscissa, Fig. 8B) for 100 ms. This was repeated at baseline, in heptanol, and after heptanol removal (washout). The end-pulse membrane potential of Cell2 (ordinate, Fig. 8B) increased in proportion to the injected current. Heptanol reversibly abrogated the rise of voltage at Cell2. Figure 8C shows the electrode voltage required to inject 100- and 300-pA currents into Cell1. Consistent with a rise in input resistance, that voltage was higher in heptanol. Figure 8D provides a summary of the input resistance calculated from the ratio of the rise voltage to the rise in current injected into Cell1. As expected, heptanol increased input resistance because higher voltage is required to drive current through partially blocked gap junctions. At very high currents (600 to 1,100 pA), the electrode voltage and hence membrane potential of Cell1 increased to nonphysiological levels (several hundred millivolts) and differences in cell layer resistance between heptanol and vehicle became insignificant (data not shown). We speculate that forcing such large currents through the preparation may have reversed heptanol blockade. Note that during these current injection experiments, the difference between electrode voltage and membrane potential of Cell1 is expected to remain small (<10 mV) because access resistance is <10 MΩ.

Diffusive transfer of LY between cells of the DVR wall. We previously showed that LY can diffuse between DVR endothelial cells (54). In this study, we measured the time required for LY to diffuse between cells. This was tested in endothelia (stripped vessels, Fig. 9A) and pericytes (intact vessels, Fig. 9B). Images were acquired every 30 ms for up to 2 min after rupture of the patch. The final images of several experiments are shown in Fig. 9C. LY diffused along the endothelial layer (multiple cell bodies, images a–g) but remained confined within pericytes (single cell body, images h–n). Interestingly, on the time scale of observation, LY remained ipsilateral within adjacent endothelial cells. In no case did it spread to the contralateral wall implying that endothelial gap junctions accommodate diffusion along the vessel axis more readily than across the axis, in the transverse direction.

Fig. 5. Dual patch clamp of endothelia and pericytes on the DVR wall. A and B: schematic depictions of dual patch clamp of DVR endothelia (dark blue) or abluminal pericytes (light blue) on the DVR wall. In each case, one cell (Cell1) is voltage clamped while 0 current-clamp is performed at Cell2 to record membrane potential. C: configurations in A and B were used to subject Cell1 (pericyte or endothelial cell) to a depolarization from −80 to −40 mV for 15 ms (above graph). Sampling of membrane potential was performed at the maximum possible rate of 250 kHz at Cell2 (ordinate). The data show means ± SE reduced for display by averaging 10 adjacent points (i.e., reduced to 25 kHz). Resting potential at Cell2 was lower in the case of the pericyte-to-pericyte recordings and the rate of depolarization of Cell2 was slower (τ = 34 ± 10 ms, n = 7 vessels, 7 rats) than with endothelial-to-endothelial studies (τ = 10 ± 2.9 ms, n = 5 vessels, 5 rats). D: rapid sampling (250 kHz) of the data in C permits expansion of the recordings near the initiation of the depolarizing pulse to yield microsecond resolution. The example provided is of endothelial-to-endothelial transmission but is also typical of pericyte-to-pericyte recordings. A delay of ∼16 μs is observed between the onset of depolarization at Cell1 and the rise of membrane potential at Cell2.
We quantified the time required for diffusion of LY to adjacent endothelia. A single example is illustrated in Fig. 10, A–D. In Fig. 10A, early images after rupture show two endothelial cell bodies below and three cell bodies above the site of gigaseal rupture. Those cells are labeled with numbers that correspond to their flanking positions. “Region-of-interest” analysis (Fig. 10B) yields the background subtracted fluorescence of those cells as a function of time. Fluorescence of the cells that are distant from the pipette rises most slowly (Fig. 10, B–D). The time required for LY to diffuse is several seconds.
(abscissa), many orders of magnitude slower than that required for transmission of membrane potential (i.e., microseconds Fig. 5D). The time range indicated by the black bar above the abscissa of Fig. 10B is expanded in Fig. 10C. The delay between membrane rupture at Cell0 and arrival of LY at the flanking cell was quantified as the time after which fluorescence always exceeds upward deviations of the background “noise” (dashed line). This experiment was performed in several vessels by adjusting the initial focal plane to optimize view of the patch site at Cell0. As such, plausible imaging of adjacent cells is limited by the degree to which they are within focus and the magnitude of the rise in fluorescence from baseline is affected by both accumulation of LY and the focal plane. Results for endothelial cells of many vessels are summarized in Fig. 10E where quantifiable images were captured of cells that flank the patch pipette by either one, two, or three cell bodies. Delays of up to 7 s were observed.

**DISCUSSION**

DVR are \(~13\,\mu m\) microvessels derived from efferent arterioles of juxtamedullary glomeruli that supply the medulla of
the kidney with blood flow. It is generally accepted that their countercurrent arrangement accommodates urinary concentration and favors the low-oxygen tension in the medulla that contributes to its vulnerability (16, 36, 40, 44). Studies have shown that DVR pericytes are vasoactive and can respond to many influences but investigations necessarily rely on explanted vessels (34, 43) or isolated tissue (12, 44) because the outer medulla of the kidney is enveloped by the cortex and inaccessible, in vivo. Explantation raises many questions about the integrity of the preparations and the extent to which properties have been altered. These studies have relied on patch clamp of explanted DVR. Time delays between isolation and experimentation might be accompanied by trafficking of connexins out of the cell membrane, particularly when temperature is temporarily raised during the enzymatic digestion that is required to make gigaseals feasible; moreover, that digestion might affect extracellular domains of membrane proteins. Ambient oxygen concentration to which vessels are exposed after explant is much higher than that of the renal medulla. Modification of cell signaling or generation of reactive oxygen species may occur. Finally, although our buffers are hypertonic to plasma, they do not replicate the osmotic environment or solute content of the outer medulla. Despite these concerns, our findings of syncytial properties between renal pericytes are similar to parallel observations in the retina where cell-to-cell transmission has also been found (32, 46, 56).

Many studies of the physiological consequences of altered medullary perfusion favor the interpretation that control of DVR tone involves paracrine interactions between pericytes, endothelia, and adjacent nephrons. As with other microvessels, our study of the DVR endothelium reveals robust coupling by gap junctions (54). In this study, we used the fluorescent voltage dye di-8-ANEPPS to corroborate endothelial coupling. In vessels where pericytes had been removed, voltage clamp-induced depolarization of a single cell resulted in depolarization of distant cells along the vessel axis with responses that are sensitive to gap junction blockade (heptanol, blue), higher voltages were needed to pass current through the cell layer. D: summary of input resistances at baseline and in heptanol (blue), calculated from the ratio of the increase in voltage to the increase in current of experiments exemplified in C. **P < 0.01 heptanol vs. vehicle.
result favors the interpretation that myo-endothelial coupling is absent or of high resistance but this requires cautious interpretation. As with other fluorescent probes, the signal derived from simultaneous introduction into pericytes and endothelia yields a predominantly endothelial signal. Moreover, a slow spread of charge from pericyte to endothelial layer might exist but requires a longer time frame to yield observable results.

In the past, the existence of cell-to-cell coupling of [Ca\(^{2+}\)]\text{CYT}\) signaling and membrane potential between DVR pericytes and endothelium had been uncertain. We provided evidence that pericytes communicate with one another along the DVR wall. Mechanical deformation of a pericyte surface by a microscopic fluid stream depolarized distant pericytes and elevated their [Ca\(^{2+}\)]\text{CYT}\) concentrations without inducing similar changes in the endothelium (61). Independence of [Ca\(^{2+}\)]\text{CYT}\) signaling in pericytes and endothelia has also been observed during agonist stimulation with angiotensin II (ANG II). ANG II exposure leads to oscillatory [Ca\(^{2+}\)]\text{CYT}\) elevations in DVR pericytes that precede and are synchronized with spontaneous transient inward Cl\(^{-}\) currents. During those pericyte oscillations, endothelial [Ca\(^{2+}\)]\text{CYT}\) declines monotonically (39, 55). ANG II may tend to reduce transmission between cells, as it does in the retina (56), but this is untested in DVR. In contrast to the independence of [Ca\(^{2+}\)]\text{CYT}\), agonist-induced membrane potential varies in parallel after whole vessel exposure to ANG II (depolarizations) or bradykinin (hyperpolarizations) (48). Those results might be a consequence of independent receptor-mediated signaling or because myo-endothelial coupling spreads electrical signals between the two cell layers (17). We speculated that membrane potential may be shared between pericytes and endothelium to account for spread of mechanosensitive responses to distant cells (61). The evidence presented herein tends to contradict that hypothesis; instead, it favors the notion that pericytes can function as an independent syncytium. The extent of myo-endothelial coupling in DVR has not been rigorously tested and dedicated studies will be needed. Electrical communication between pericytes is rapid (Fig. 5) and depolarization of a pericyte does not result in rapid voltage changes in the endothelium (Fig. 4C). With respect to myo-endothelial communication of membrane potential, studies in other vascular beds have yielded variable results (1, 51).

In this study, we directly confirmed that DVR pericytes are electrically coupled (Figs. 5–8). That finding is consistent with the recent observation that mechanical stimulation spreads instantaneously from pericyte to pericyte along the DVR wall (see Fig. 1 of Ref. 61). Cable analysis is often used to simulate neurovascular electrical coupling to calculate “length constants” over which electrical effects originating in one cell can exert vicinal influence on its neighbors. That approach treats the nerve or vessel as a cable with uniform properties, rather than assigning discrete resistors and capacitors to an equivalent circuit (47). Given the limited number of cell separations involved in these short-vessel segments, application of cable analysis seems inappropriate. Nonetheless, some observations
Nephrons and DVR are mechanosensitive and can address the plausible range of conducted responses. Over several hundred microns, mechanical stimulation (61) or voltage-clamp depolarization (Figs. 3–7) changes modified membrane potential or $[\text{Ca}^{2+}]_{\text{CYT}}$ along the DVR wall. In the rat, the medulla of the kidney is ~5–7 mm long, 2 mm of which is the outer medulla (26, 42). Based on that, communication of electrical signals between juxtamedullary efferent arterioles or sympathetic nerves to outer medullary vascular bundles via DVR mural syncytia seems plausible. Conversely, release of paracrine mediators in the vicinity of outer medullary vascular bundles might affect upstream vascular diameter through conduction vasodilatory or constrictor responses. Neural control of the renal medulla has been reviewed by others (15, 16). Sympathetic innervation and its association with pericytes may vary with medullary depth (3, 10, 13, 18). The present studies suggest that a depolarizing impulse originating from neural activity might extend its range of influence considerably by conduction within the DVR wall.

Given the inaccessibility of vascular bundles in vivo, the physiological consequences of mural transmission through DVR syncytia are speculative and may be difficult to rigorously test. Nephrons and DVR are mechanosensitive and can release vasoactive mediators in response to shear and stretch (4, 14, 53, 60, 61). Isolated vessel and isolated tissue preparations eliminate luminal shear as well as flowing plasma and red blood cells that provide a sink for nitric oxide and other molecules (7–9, 12, 34). As such, tonic signaling may be altered and we may be introducing artifacts. While conservative interpretation is always appropriate, the possibility that DVR might transmit electrical signals into or out of the medulla to affect vasomotion, pericyte vasoactivity, or release of signaling molecules seems inescapable. Investigations in the renal cortex where in vivo access is more tractable have revealed remarkable spatial synchronizations. For example, tubuloglomerular feedback is associated with sustained oscillations of single nephron glomerular filtration rates and glomerular blood flow. Those responses involve depolarization of smooth muscle along the afferent arteriole and propagation of those signals couples the behavior of neighboring nephrons (27, 30, 52). Remarkable new studies with laser speckle contrast imaging have revealed patterns of synchronization that spread to several nephrons observable on the cortical surface (24, 50). Although juxtamedullary nephrons that feed efferent arterioles cannot be visualized by similar imaging, the possibility

Fig. 10. Endothelial spread of LY along the DVR wall. A: image shows spread of LY from the pipette to Cell0 via a ruptured patch. LY also spreads to flanking cells, labeled 1, 2, or 3 corresponding to their sequential locations with respect to Cell0. B: LY fluorescence from the individual cell bodies labeled in A is presented as a function of time. Fluorescence from the patched cell rises most rapidly (Cell0, black) while that in adjacent cells lags behind. The black bar above the abscissa indicates the region expanded in C. C: expanded region from B illustrates the delay required for LY fluorescence to rise in flanking cells (Cell1, Cell2, Cell3). Several second lapses before LY remains consistently above background (dashed line). The first time point beyond which all background subtracted fluorescence exceeds 0 is quantified as the “delay.” D: sequential images show an example of the spread of LY from Cell0 to flanking endothelial cells. Image 0 is the time of membrane rupture. Image 675 (20.25 s after rupture) is that reproduced as the example in E. Summarizes the delay that precedes the appearance of LY fluorescence in sequential cells that flank the patched cell (Cell0) by 1, 2, or 3 cell bodies (Cell1, Cell2, Cell3, respectively). Some vessels are counted more than once (e.g., Cell1 has 15 points) because Cell1 sometimes exists twice, on both sides of the pipette.
that efferent arterioles spread analogous signals over macroscopic distances to affect medullary perfusion is a logical consideration. Finally, the observations that voltage-gated Na⁺ channels (Nav1.3) and voltage-gated Ca²⁺ channels are expressed by DVR pericytes suggest a channel architecture similar to that required to propagate action potentials (21, 28, 57, 59). That raises the question of paced vasomotion within medullary vascular bundles downstream of neural activity in the cortex.

The ability of DVR pericytes to conduct a depolarizing pulse or mechanically induced signal contrasts with the limited ability of LY to diffuse between adjacent cells (Figs. 7 and 8). Poor LY diffusion has been observed in the smooth muscle layer of other microvessels and might be accounted for by steric hindrance or a tendency of LY, per se, to close the connexon channels that comprise the gap junctions. LY may not be the optimal probe for investigation of steric hindrance in smooth muscle cells such as DVR pericytes (22, 29). When an abrupt increase of membrane potential is imposed by voltage clamp, the pericyte layer depolarizes more slowly than the endothelial layer (Fig. 5). That implies, of the two syncytia, that pericytes are likely to be of higher resistance. Past measurement of capacitance transients in the two cell types led to a similar conclusion (54). The data obtained with videomicroscopy of LY at high framing rates (30 ms/image) in endothelia show that resistance to diffusion is low and has spatial orientation. Within 2 min of observation, LY only diffused to ipsilateral cells that flank the patch pipette without reaching the contralateral wall (Fig. 7). Given that DVR endothelia surround the lumen like staves of a barrel (38, 41), endothelial connexons may be organized to favor axial rather than transverse diffusion of signals between adjacent cells. LY is a sulfated probe with negative charge. Further investigation of pericyte-to-pericyte or myo-endothelial diffusion using tracers with neutral or positive charge is warranted.

We quantified the rate of spread of LY between endothelial cells using videomicroscopy (Figs. 7 and 8). LY required several seconds to diffuse to cells located ~100 μm away from the site of its introduction. That finding contrasts with a rapid spread of voltage between endothelial cells that can occur within microseconds (Fig. 5). A microsecond time scale cannot be accounted for by cell-to-cell diffusion of a signaling molecule. Instead, modulation of the electrical field, akin to that involved in neural transmission, is likely to be the mechanism. Outer medullary DVR are inaccessible in vivo so that these studies require isolation of vessel segments from vascular bundles that are typically 500 μm long. Those short lengths constrain spatial separation of two electrodes. The need, during dual electrode patch clamp, to form and maintain two gigaseals within the visual field of the investigator is also a limiting factor. The consequence is that separation of electrodes to increase delay time to improve accuracy of measurement is technically limited. Despite those problems, delays were observable when a sampling rate of 250 kHz was used but temporal resolution remained limited to ±4 μs (Fig. 5D). As a consequence, calculations of conduction velocity are approximate but probably exceed 10 m/s. Hence, electrical signals could travel along the DVR wall on a time scale that, relative to physiological events, is instantaneous, as was observed (61).

REFERENCES
