Calcium wave of tubuloglomerular feedback

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Calcium wave of tubuloglomerular feedback. Am J Physiol Renal Physiol 291: F473–F480, 2006. First published February 21, 2006; doi:10.1152/ajprenal.00425.2005.—ATP release from macula densa (MD) cells into the interstitium of the juxtaglomerular (JG) apparatus (JGA) is an integral component of the tubuloglomerular feedback (TGF) mechanism that controls the glomerular filtration rate. Because the cells of the JGA express a number of calcium-coupled purinergic receptors, these studies tested the hypothesis that TGF activation triggers a calcium wave that spreads from the MD toward distant cells of the JGA and glomerulus. Ratiometric calcium imaging of in vitro microperfused isolated JGA-glomerulus complex dissected from rabbits was performed with fluo-4/fura red and confocal fluorescence microscopy. Activation of TGF by increasing tubular flow rate at the MD rapidly produced a significant elevation in intracellular Ca2+ concentration ([Ca2+]i) in extraglomerular mesangial cells (by 187 ± 45.1 nM) and JG renin granular cells (by 281.4 ± 66.6 nM). Subsequently, cell-to-cell propagation of the calcium signal at a rate of 12.6 ± 1.1 μm/s was observed upstream toward proximal segments of the afferent arteriole and adjacent glomeruli, as well as toward infraglomerular elements including the most distant podocytes (5.9 ± 0.4 μm/s). The same calcium wave was observed in nonperfusing glomeruli, causing vasoconstriction and contractions of the glomerular tuft. Gap junction uncoupling, an ATP scavenger enzyme cocktail, and pharmacological inhibition of P2 purinergic receptors, but not adenosine A1 receptor blockade, abolished the changes in [Ca2+]i, and propagation of the calcium wave. These studies provided evidence that both gap junctional communication and extracellular ATP are integral components of the TGF calcium wave.

ATP; adenosine; purinergic receptors; gap junction; fluo-4; fluorescence microscopy; podocyte

The macula densa (MD) cells of the juxtaglomerular (JG) apparatus (JGA) detect changes in distal tubular flow rate and generate signals to control glomerular filtration rate (tubuloglomerular feedback, TGF) and renin release (3, 40). There seems to be a consensus that the TGF mechanism involves ATP release from MD cells into the JGA interstitium (3, 19), its degradation to adenosine (6, 35), and the actions of ATP, adenosine, or both on afferent arteriole (AA) vascular smooth muscle cells (40) to cause vasoconstriction. Several studies support the importance of both ATP and adenosine in JGA function. P2X1 purinergic receptor-deficient mice have impaired autoregulatory responses (15), whereas adenosine A1 receptor-knockout mice lack TGF responses (44). Also, very recent works that found impairment of TGF in ecto-5′-nucleotidase/CD73-deficient mice (6) and with pharmacological inhibition of the enzyme (35) further support the existence of a functionally active ATP dephosphorylating enzyme cascade in the JGA and the mediator role of adenosine in TGF.

With either ATP acting on P2X or P2Y receptors or adenosine activating A1 receptors as the primary mediator of TGF, calcium is the most likely unifying downstream signaling mechanism (11, 13). The intracellular calcium concentration ([Ca2+]i) of vascular smooth muscle cells has long been recognized as the predominant second messenger in the regulation of vascular tone (38). Also, [Ca2+]i in renin granular cells is an important regulator of renin release (51). Cells of the JGA and mesangium express a variety of P2X and P2Y receptors (14, 15, 26, 41), and the JG portion of the AA is highly abundant in A1 receptors as well (26, 40, 49). Consistent with this structural organization, the generation of a calcium signal and its propagation in the JGA during TGF activation have been hypothesized. Recent studies using cell culture models demonstrated cell-to-cell calcium signaling, an extracellular ATP-dependent calcium wave between JG granular cells that inhibited renin secretion (51). Similarly, a gap junction-mediated intercellular calcium wave observed in mesangial cells in culture resulted in coordinated cell contraction (22, 52).

The effector cells of TGF are most likely the contractile cell types of the JGA, including AA smooth muscle cells and extraglomerular mesangial cells, although the putative calcium signal may propagate toward infraglomerular elements as well. Cells of the JGA, except MD cells, are interconnected by gap junctions (45), which are ideal for the fast transmission of vasoconstrictor signals. Both extra- and infraglomerular mesangial cells, as well as podocytes, are equipped with contractile machinery and were suggested to control glomerular filtration (9, 22, 45).

Accordingly, the purpose of these studies was to visualize, in real time and in situ, the putative intercellular propagation of calcium signals during TGF from the MD area to the JGA and beyond. The following main questions were addressed: 1) Is there evidence for a calcium wave spreading through the mesangial cell field to the AA smooth muscle cells? 2) What other JGA or infraglomerular cells have alterations in [Ca2+]i during TGF signaling? 3) Is [Ca2+]i signal spreading attributed to gap junctional coupling or extracellular signaling molecules?

MATERIALS AND METHODS

In vitro isolated and microperfused AA-JGA-glomerulus. A superficial AA with its glomerulus and attached distal tubule containing the MD was microdissected from kidneys of female New Zealand White rabbits (500 g, Irish Farms, Norco, CA). All animal protocols were approved by the Institutional Animal Care and Use Committee of the University of Southern California.

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approved by the Institutional Animal Care and Use Committee at the University of Southern California. Briefly, the dissection medium was prepared from DMEM (DME mixture F-12, Sigma) with the addition of 1.2 g/l NaHCO3 and 3% fetal bovine serum (Hyclone). Before use, this solution was aerated with 95% O2-5% CO2 for 45 min, and pH was adjusted to 7.4. The arteriole was perfused with a modified Krebs-Ringer-HCO3 buffer containing (mM) 115 NaCl, 5 KCl, 25 NaHCO3, 0.96 NaH2PO4, 0.24 Na2HPO4, 1.2 MgSO4, 2 CaCl2, 5.5 D-glucose, and 10 μM 1-arginine, and perfusion pressure was maintained at ~50 mmHg (1 psi) throughout the experiment. The tubular perfusate was an isosmotic, low-NaCl-containing Ringer solution consisting of (mM) 10 NaCl, 135 NaHCO3, 0.96 NaH2PO4, 0.24 Na2HPO4, 2 CaCl2, 5.5 D-glucose, and 100 μM 1-arginine, and perfusion pressure was maintained at ~50 mmHg (1 psi) throughout the experiment. The tubular perfusate was an isosmotic, low-NaCl-containing Ringer solution consisting of (mM) 10 NaCl, 135 NaHCO3, 0.96 NaH2PO4, 0.24 Na2HPO4, 2 CaCl2, 5.5 D-glucose, and 10 HEPES. The tubule segment was cannulated and perfused at a baseline rate of ~2 nl/min, or 5 psi. Tubuloglomerular feedback (TGF) was stimulated by increasing tubular perfusion rate to 20 nl/min, or 5 psi.

**Calibration of tubular perfusion pressure to flow.** Glass microperfusion pipettes used in the experiments were filled with the same modified Ringer solution but also containing the fluorescent dye Lucifer yellow (5 mM; Molecular Probes). With the help of a micromanipulator (MP 225M, Sutter Instrument, Novato, CA), the tip of the perfusion pipette was placed within a drop of mineral oil. Micropipet perfusion was performed for 1 min, using a custom-made microperfusion apparatus (Vestavia Scientific, Vestavia, AL) and applying various perfusion pressures between 1 and 10 psi. Confocal microscopy fluorescence (excitation and detection of Lucifer yellow at 458 and 536 nm, respectively) and z-sectioning confirmed that the shape of the Ringer solution perfused into the oil was a uniform sphere and its volume was stable. Volume of the perfused droplet was calculated by measuring the radius of the sphere from the fluorescence images and was plotted against perfusion pressure (Fig. 1).

**Confocal laser-scanning fluorescence microscopy.** Calcium imaging was performed with a Leica TCS SP2 AOBS MP confocal microscope system (Leica Microsystems, Heidelberg, Germany). A Leica DM IRE2 inverted microscope was powered by a blue Ar 488-nm, 20-mW laser for these studies. Images were collected in time series (zxy, 2 s per frame) with the Leica LCS imaging software, and for ratiometric calcium imaging the fluorescent pair fluo-4 (excitation at 488 nm, emission at 520 ± 20 nm) and fura red (excitation at 488 nm, emission at >600 nm) was used. All experiments were performed with the same instrument settings (laser power, offset, gain of both detector channels). Fluorescence intensity (12 bit) and vascular and glomerular diameter values were measured with the Leica LCS imaging software’s (LCS 2.61.1537) Quantification Tools. Techniques and advantages of this ratiometric pair for confocal fluorescence microscopy and their application in imaging renal tissues were reviewed recently (20, 29, 46).

The preparations were loaded with the dyes by adding fluo-4 AM and fura red AM (both at 10 μM; Invitrogen) dissolved in dimethyl sulfoxide to both the tubular and arteriolar perfusate. Loading required ~20 min, after which fluorescent dyes were removed from both lumens. A ~20-min incubation of the preparation with the control perfusion solutions was allowed to permit stabilization of fluorescent signals. An equal distribution of fluo-4 and fura red within the cells is demonstrated in Fig. 2, A and B. Fluorescence of fluo-4 increased (from A to C), whereas fura red fluorescence decreased (from B to D) in response to intracellular Ca2+ concentration ([Ca2+]i) elevations (5 μM ionomycin and 1.5 mM CaCl2). Different cell types of the glomerulus (E, endothelium; M, mesangium; P, podocytes) were identified based on anatomic considerations. Bar is 20 μm. E: although the baseline [Ca2+]i levels were different, all cells produced identical fluo-4-to-fura red ratios under saturating calcium conditions (Rmax, from the same cell types and conditions as shown in A–D). F: in situ intracellular calibration of the fluo-4-fura red mixture. Fluorescence ratios were recorded in various Caii calibration solutions. Ka was 506 nM.

**Fig. 1.** Linear relationship between the applied perfusion pressure and calculated tubular flow rate. Baseline tubular perfusion of ~2 nl/min was achieved by perfusing between 1 and 2 psi. Tubuloglomerular feedback (TGF) was stimulated by increasing tubular perfusion rate to 20 nl/min, or 5 psi.

**Fig. 2.** Calibration of fluo-4 (green) and fura red (red) fluorescence. The two fluorophores distributed equally in the cytoplasm, with higher accumulation in cell nuclei (A and B). Fluorescence of fluo-4 increased (from A to C), whereas fura red fluorescence decreased (from B to D) in response to intracellular Ca2+ concentration ([Ca2+]i) elevations (5 μM ionomycin and 1.5 mM CaCl2). Different cell types of the glomerulus (E, endothelium; M, mesangium; P, podocytes) were identified based on anatomic considerations. Bar is 20 μm. E: although the baseline [Ca2+]i levels were different, all cells produced identical fluo-4-to-fura red ratios under saturating calcium conditions (Rmax, from the same cell types and conditions as shown in A–D). F: in situ intracellular calibration of the fluo-4-fura red mixture. Fluorescence ratios were recorded in various Ca2+ calibration solutions. Ka was 506 nM.
made permeable to calcium with ionomycin (5 μM), and ambient solutions were applied and equilibrated with [Ca2+]i between 0 (with 2 mM EGTA) and 1.5 mM (Fig. 2F). The operational Kd value for the dye mixture (506 nM) was calculated with nonlinear curve fitting (GraphPad Prism). Mean Rmax and Rmin values (R under saturating and Ca2+-free conditions, respectively) were 3.85 and 0.26, respectively. R values were converted into [Ca2+]i values with the equation [Ca2+]i = Kd × (Rmax − R)/Rmax.

Materials. The following pharmacological agents were used in these experiments: 100 μM furosemide, a specific blocker of the Na-2Cl-K cotransporter; the nonselective purinergic receptor blocker suramin (50 μM); 100 μM 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), a selective adenosine A1 receptor blocker; 100 nM Nω-cyclohexyladenosine (CHA), a potent adenosine A1 receptor agonist; 25 μM α-glycyrrhetinic acid (α-GA), a gap junction uncoupling agent (all dissolved in dimethyl sulfoxide); 500 μM heptanol, a gap junction uncoupling agent; and an ATP scavenger cocktail consisting of hexokinase and apyrase (both 50 U/ml). Unless otherwise indicated, chemicals were purchased from Sigma (St. Louis, MO).

Data analysis. Data are expressed as means ± SE. Statistical significance was tested with Student’s t-test for paired samples. Significance was accepted at P < 0.05.

RESULTS

Baseline [Ca2+]i. Baseline [Ca2+]i in the different cell types of the AA-glomerulus-attached cortical thick ascending limb (cTAL) preparation was highly variable (Fig. 3A). Average [Ca2+]i in the MD plaque was the lowest (80.9 ± 23.9 nM, n = 11) among all cell types studied and did not change significantly in response to TGF activation applied in these experiments (increased to only 113.7 ± 34.7 nM). Epithelial cells of the cTAL (157.8 ± 53.2 nM, n = 8) and podocytes (273.2 ± 56.6 nM, n = 10) had the next lowest baseline [Ca2+]i, whereas JG cells (350.9 ± 52.9 nM, n = 8) and endothelial cells (363.1 ± 49.6 nM, n = 15) had baseline [Ca2+]i, levels similar to those in contractile cells: extraglomerular (376.7 ± 42.9 nM, n = 18) and intraglomerular (403.5 ± 19.9, n = 17) mesangial cells and vascular smooth muscle cells (461.7 ± 54.9 nM, n = 17). Interestingly, baseline [Ca2+]i showed oscillations in most cell types, including some cells of the cTAL epithelium. Oscillations were usually high in magnitude and irregular, except the regular [Ca2+]i transients produced every 45–50 s in podocytes (Fig. 3B).

Salt- vs. flow-induced TGF. Increasing the rate of constant 10 mM NaCl-containing tubular perfusion from 2 to 20 nl/min induced a sustained, reversible, and reproducible TGF-mediated vasoconstriction of the terminal AA (ID reduced by 46.1 ± 4.4%) and a reduction in the diameter of the glomerular tuft by 7.3 ± 0.6%. These morphological changes were associated with a significant increase in [Ca2+]i in most cells of the AA and glomerulus (Figs. 4 and 5). For example, [Ca2+]i in vascular smooth muscle cells of the AA increased by 223.7 ± 21.5 nM (n = 14).

In some experiments (n = 4) the cTAL was completely removed from around the MD plaque, so the apical surface of
MD cells was directly accessible from the bath with a perfusion pipette (Fig. 6A). High flow (20 nL/min) applied directly to the MD area caused no visible changes in the morphology of MD cells (not shown). However, it produced AA vasoconstriction and calcium responses (Fig. 6B) identical to intact tubule perfusion as described above. The preparation showed no signs of desensitization (Fig. 6B).

Interestingly, smooth muscle cells of the efferent arteriole (EA) produced a similar response: [Ca\(^{2+}\)]\(_i\), increased by 224.9 ± 60.9 nM (n = 4) after TGF activation. Furosemide (100 μM) added to the tubular perfusate abolished all the morphological and calcium changes induced by the increased flow rate. For example, the change in [Ca\(^{2+}\)]\(_i\) in vascular smooth muscle cells of the AA was only −8.1 ± 19.1 nM (see Fig. 10).

As with flow-induced TGF, increasing [NaCl] at the MD from 10 to 80 mM induced a significant vasoconstriction of the AA (ID reduced by 42.6 ± 8.1%, reduction in the diameter of the glomerular tuft by 4.1 ± 0.9%, and increases in [Ca\(^{2+}\)]\(_i\), (Fig. 5). However, the magnitude of these changes was smaller than that observed with flow-induced TGF. For example, [Ca\(^{2+}\)]\(_i\) in vascular smooth muscle cells of the AA increased by only 107.5 ± 15.5 nM (P < 0.05, compared with the flow-induced change; Fig. 5).

Real-time visualization of TGF calcium wave. Activation of TGF by increasing tubular flow rate at the MD rapidly (within 2 s) produced a significant [Ca\(^{2+}\)]\(_i\), elevation in extraglomerular mesangial cells and the JG renin granular cells closest to the MD (Fig. 7). Subsequently, cell-to-cell propagation of the calcium signal was observed from the MD region toward both the proximal AA (within 10 s) and all intraglomerular cell types, including the most distant podocytes (within 20 s; Fig. 7). Videos of the same preparation showing the fluo-4 signal and differential interference contrast image are available as supplemental data contained in the online version of this article. This calcium wave appeared to propagate upstream toward the proximal AA faster than in the intraglomerular direction, at a rate of 12.6 ± 1.1 and 5.9 ± 0.4 μm/s, respectively (n = 8 each, P < 0.05). Propagation of the high [Ca\(^{2+}\)]\(_i\), from vascular smooth muscle cells of the AA into the underlying endothelium was also noted (Fig. 7).

A TGF-induced calcium wave of similar speed and magnitude was observed in nonperfusing glomeruli (Fig. 8). Elevations in [Ca\(^{2+}\)]\(_i\), were associated with contractions of both the AA and the glomerular tuft, causing significant reductions in the AA and glomerular diameter (not shown).

Propagation to adjacent glomeruli. In some cases, a terminal interlobular artery giving rise to two separate AAs and glomeruli was dissected and micropipetted. Stimulating only one MD by increased tubular flow induced an equally strong TGF in both glomeruli (diameter of the glomerular tuft reduced by 5.6 ± 1.1% in the parent glomerulus and by 5.4 ± 0.8% in the adjacent glomerulus; n = 4). Calcium imaging allowed direct visualization of the communication between adjacent glomeruli through their AAs (Fig. 9). TGF activation triggered the propagation of a calcium wave not only into the parent glomerulus, but also upstream to the adjacent AA and then downstream to the other glomerulus (an ~100-μm distance) within 10 s. The magnitude of calcium changes in both AAs and glomeruli was similar to that of the single-glomeruli [Ca\(^{2+}\)]\(_i\) changes described above. Consequently, the two adjacent glomeruli produced parallel TGF responses, AA vasoconstriction, and contraction of the glomerular tuft (Fig. 9).

Role of ATP. Pharmacological experiments were performed to identify the mechanism of cell-to-cell propagation of the TGF calcium wave. Addition of the nonselective P2 purinergic receptor blocker suramin (50 μM) to the AA perfusate and bathing solution completely abolished the increases in the terminal AA [Ca\(^{2+}\)]\(_i\), and consequently the calcium wave and morphological changes of TGF (Fig. 10). Furthermore, preincubation of the bathing solution with an ATP scavenger cocktail consisting of apyrase and hexokinase (both 50 U/ml) produced a similar inhibitory effect (Fig. 10). After a 10-min washout of these enzymes from the bath, calcium and morphological changes during TGF were restored (AA [Ca\(^{2+}\)]\(_i\), increased by 168.6 ± 52.1 nM, AA diameter reduced by 46.1 ± 3%). Interestingly, the addition of DPCPX (100 μM), a selective adenosine A1 receptor blocker, to the AA perfusate and bathing solution had no effect (Fig. 10). Effective blockade of A1 receptors was confirmed by subsequent addition of the A1 receptor agonist CHA (100 nM) to the bathing solution. In the presence of DPCPX, CHA produced no significant change in AA diameter (Δ = −1.2 ± 3.5%, n = 6), whereas in control
preparations 100 nM CHA caused strong AA vasoconstriction (ID reduced by 47.7 ± 4.5%).

Role of gap junctions. Preparations were preincubated for 5 min with the gap junction uncoupler heptanol (500 μM) or α-GA (25 μM) added to the AA perfusate and bathing solution. In the presence of heptanol, TGF activation still caused a significant vasoconstriction of the terminal AA (ID reduced by 49.7 ± 4.0%). TGF activation induced elevations in [Ca²⁺], only in the terminal AA (Fig. 10), in the two or three cells closest to the MD. However, the calcium wave dissipated beyond these cells, and there were no significant changes in [Ca²⁺], in the proximal AA or in the glomerulus. Intraglomerular mesangial cells, measured ~ 20 μm from the MD, produced only minor changes in baseline [Ca²⁺], during heptanol treatment (increased by 58.4 ± 7.9 nM compared with control; Δ = 259.3 ± 26.7 nM, P < 0.05). Accordingly, TGF activation failed to induce contraction of the glomerular tuft and reduce the glomerular diameter (Fig. 10). Preincubation with the structurally unrelated gap junction inhibitor α-GA produced an even more potent inhibition of the calcium wave. In addition to blocking glomerular changes during TGF, α-GA significantly reduced [Ca²⁺] elevations in the terminal AA (Fig. 10). Consequently, the AA vasoconstriction was significantly reduced (ID reduced by 21.4 ± 4.4% compared with control; Δ = 54.6 ± 12.8%).

DISCUSSION

In the present study, TGF-induced calcium signals were analyzed in all cells of the intact, in vitro microperfused AA-JGA-glomerulus complex. For the first time, confocal fluorescence imaging techniques with high temporal and spatial resolution allowed real-time visualization of the putative calcium wave of TGF. A rapid cell-to-cell propagation of the calcium wave was observed to originate from the MD area and travel upstream toward proximal segments of the AA and adjacent glomeruli, as well as toward all intraglomerular elements, including the most distant podocytes. Changes in [Ca²⁺], were associated with the classic morphological feature of TGF: the strongest vasoconstriction was found in the terminal, intraglomerular AA and propagated upstream to proximal segments (24, 30). In addition, these studies provided functional evidence for previous speculations (45) that all cells of the glomerulus actively participate in TGF by contracting the glomerular tuft, thereby helping to reduce the rate of glomerular filtration. Moreover, the unexpected finding that the calcium wave of TGF was mediated by extracellular ATP provides further support that ATP itself is directly involved in TGF (3, 15, 19, 25–27), and not only through its breakdown to adenosine (6, 35, 40).

These experiments used increases in tubular flow rate as the classic experimental stimulus of TGF activation (2, 4). Rates of tubular perfusion were calibrated (Fig. 1), and the applied rate of 2–20 nl/min is in the physiological range of highest TGF sensitivity (4). This increase in tubular flow ultimately produced a TGF response similar to that seen with salt, although the effect of flow was more pronounced (Fig. 5). Also, it was completely inhibited by furosemide (Fig. 10), a hallmark of the TGF mechanism. Although controversial (2), it is generally accepted that [NaCl] of the tubular fluid and not flow itself is the stimulus for TGF activation (4). The availability of salt (10 mM NaCl in the perfusate) and furosemide sensitivity in the present experiments are consistent with the paradigm (4, 22) that MD salt transport triggers TGF. The flow-induced TGF was clearly associated with an increase in tubular diameter (Fig. 7). However, in the absence of tubular distension (Fig. 6), increased flow at the MD produced the same results as with...
addresses a long-debated issue, suggesting that MD calcium and contractile responses in other cells. This finding significantly during stimuli that caused strong TGF-mediated enous ATP induces [Ca\(^{2+}\)]. This is consistent with our earlier observation (29) that exog- and receptors different from those in the AA (16). Conse- quently, the EA is believed to respond to various stimuli in a different way (14, 16, 37). However, the present studies de- pendent upon the TGF signal transmission (21, 31, 34).

Another controversial subject is the role of the EA in TGF. Vascular smooth muscle cells of the EA express ion channels and receptors different from those in the AA (16). Conse- quently, the EA is believed to respond to various stimuli in a different way (14, 16, 37). However, the present studies de- tected the propagation of the TGF calcium wave into the EA and significant elevations in EA smooth muscle cell [Ca\(^{2+}\)]. This is consistent with our earlier observation (29) that exogenous ATP induces [Ca\(^{2+}\)] elevations in both the AA and EA, evidence that opposes other reports (14). The conflicting re- sults may be due to the different experimental conditions and preparations used. In the present isolated perfused glomerulus technique, the EA was cut open and had nearly zero vascular resistance, in contrast to the juxtapamedullary nephron prepara-

An interesting point in these studies was the observation of baseline [Ca\(^{2+}\)] oscillations in both contractile and epithelial cells. The oscillations were irregular in most cells except the podocytes. In the intact, perfused glomerulus these cells consistently produced regular [Ca\(^{2+}\)] transients every 45–50 s (Fig. 3B). Future studies should clarify whether a podocyte “pacemaker-like” function participates in the oscillations of the glomerular filtration rate observed in vivo (12, 18). Podocyte foot processes possess contractile structures, which may re- spond to vasoactive hormones and thereby regulate the ultra-
filtration coefficient (K\(_f\)) (9). In fact, propagation of the TGF calcium wave to all cells of the glomerulus was associated with the contraction of the glomerular tuft (Figs. 7 and 10 and supplemental videos) and reduction in the glomerular capillary loop diameter (30). These findings suggest that mesangial cells and podocytes are active cellular effectors of the TGF mechan-

intact tubule perfusion. These observations suggest that in- creased flow and not tubular distension activates TGF in these experiments. More studies are needed on the MD flow-sensing mechanism, including the actions of furosemide, and on the effects of tubular flow rate on MD ion transport processes.

Confocal fluorescence microscopy using highly sensitive calcium fluorophores allowed the direct visualization of [Ca\(^{2+}\)] dynamics in all cells of the JGA-glomerulus. The advantages of this technology were summarized recently (29). Consistent with previous reports (21, 31), baseline [Ca\(^{2+}\)] in the MD plaque was very low compared with other neighboring cell types. Also, the average MD [Ca\(^{2+}\)] did not change significantly during stimuli that caused strong TGF-mediated calcium and contractile responses in other cells. This finding addresses a long-debated issue, suggesting that MD [Ca\(^{2+}\)] is not involved in the TGF signal transmission (21, 31, 34).

Fig. 10. Pharmacological blockade of the calcium wave. Changes in [Ca\(^{2+}\)], measured in the terminal, intraglomerular AA are shown by open columns (left y-axis) and reductions in the diameter of the glomerular tuft by filled columns (right y-axis). Effects of the Na-2Cl-K cotransporter blocker furosemide, the nonselective purinergic receptor blocker suramin, an ATP scavenger cocktail consisting of apyrase and hexokinase (apy-hexo), 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), a selective adenosine A1 receptor blocker, and heptanol and α-glycyrrhetinic acid (α-GA), gap junction uncoupling agents, are summarized; n = 6 each. *P < 0.05, compared with control (ctrl) TGF response. Effects of tubular flow rate on MD ion transport processes. More studies are needed on the MD flow-sensing technique, the EA was cut open and had nearly zero vascular resistance, in contrast to the juxtapamedullary nephron preparation (14).

Fig. 9. Visualization of the calcium wave propagation to adjacent glomeruli. Simultaneous microperfusion of 2 adjacent AAs (AA1 and AA2) and their glomeruli (G1 and G2) through the common terminal interlobular artery (ILA). Only the left MD was cannulated and perfused and stimulated by increased tubular flow. A: transmitted light (DIC) image. B and C: fluo-4 fluorescence images before (B) and 10 s after (C) TGF activation. In addition to increases in [Ca\(^{2+}\)], note the reduction in the diameter of both glomeruli. Bar is 20 μm.
Podocytes possess a variety of P2 purinergic receptors (9), and ATP mediation of the cell-to-cell calcium wave may be the mechanism that podocytes use to play an integral role in the glomerular contractile machinery despite the lack of physical connection to endothelial and mesangial cells. The present studies confirmed that both gap junctions and extracellular ATP are important components of the TGF calcium wave (Fig. 10). These findings are consistent with the view (1, 7) that connexins (gap junctions) are not involved directly in the intercellular diffusion of the calcium wave but enhance ATP release and in this way intensify the extracellular ATP-dependent calcium wave.

The finding that the TGF calcium wave was intact in the nonperfusing glomerulus (Fig. 8) indicates that its propagation to the proximal AA does not depend on hemodynamic changes. According to the “ascending myogenic autoregulation” theory (23), the strong vasoconstriction in the terminal AA increases intravascular pressure and induces a myogenic response in proximal AA segments. This mechanism may contribute to the increased vascular resistance initiated by the propagating calcium wave during TGF.

Propagation of the TGF calcium wave to adjacent glomeruli along the common interlobular artery (Fig. 9) is consistent with the nephron-nephron interaction observed in vivo (12, 18). Also, a conducted calcium response was recently found in preglomerular vessels in vitro (39). The coordinated regulation of glomerular filtration in neighboring glomeruli may be another important function of the TGF calcium wave. It should also be noted that the values of calcium wave velocity measured in the present study are consistent with other reports on the dynamics of TGF (5, 8, 17, 48). Propagation of the high \([Ca^{2+}]\) from AA smooth muscle cells to the underlying endothelium was observed in the present studies (Fig. 7), similar to that observed in a recent preliminary report (47). This phenomenon may help to balance the TGF vasoconstriction by triggering endothelium-derived vasodilator mechanisms.

Pharmacological inhibition of P2 purinergic receptors, but not adenosine A1 receptor blockade, completely abolished the changes in \([Ca^{2+}]\), and blocked propagation of the calcium wave (Fig. 10). Lack of an adenosine-mediated component in the TGF calcium wave is an unexpected finding. On the basis of several recent studies, adenosine has been identified as the key mediator of TGF (6, 35, 40, 44), and the adenosine A1 receptor that mediates the vasoconstrictive effect is coupled to calcium, at least in the mouse (11). However, our findings suggest that the mediator of the TGF calcium wave is extracellular ATP rather than adenosine. It is not clear why these studies failed to detect the involvement of adenosine. Normal TGF responsiveness and the actions of adenosine depend on the availability of other autacoids including angiotensin II in sufficiently high concentrations (40). In this regard, the isolated, in vitro double-perfused JGA preparation is not comparable to in vivo models. Nevertheless, several points in the present study favor the direct role of ATP, as suggested by other investigators (3, 15, 19, 25–27). Effective blockade of the adenosine A1 receptor with DPCPX, a specific inhibitor (50), had no effect on the calcium responses or glomerular contraction (Fig. 10). Supporting these data, in situ hybridization failed to detect A1 receptor mRNA in the glomerular tuft (49), although the terminal AA was intensely labeled. In contrast, the nonselective P2 receptor antagonist suramin in the present studies completely abolished the calcium wave, and there was no indication of an additional component. The ATP scavengers apyrase and hexokinase had an effect similar to that of suramin. These enzymes produce ADP and AMP from ATP (43) and therefore facilitate ATP degradation to adenosine. However, this enzyme cocktail abolished, rather than stimulated, the TGF calcium wave, a finding that is consistent with a role for extracellular ATP rather than adenosine. Also, it was reported that ATP causes a significant vasoconstriction of the rabbit terminal AA in the presence of DPCPX (50). This finding is consistent with the direct involvement of ATP in the TGF calcium wave. It is possible that adenosine acts differently on the A1 receptor in rabbit tissue and may be preferentially coupled to adenylate cyclase and cAMP (26) as opposed to PLC and calcium (11). In fact, adenosine causes only minor elevations in the rabbit AA \([Ca^{2+}]\), compared with the significant effect of ATP (10).

These studies did not address the specific subtypes of P2X or P2Y receptors responsible for initiation of the calcium wave. Several P2 receptor subtypes are expressed in the A1-JGA-glomerulus complex (14, 15, 41), and the present studies intentionally used suramin, a nonspecific P2 antagonist, to block all P2 receptors. However, reproducibility of the TGF calcium wave (Fig. 6B) suggests the involvement of a nondesensitizing P2 receptor subtype. Likewise, it is well established that sustained elevations in \([Ca^{2+}]\) involve the activation of voltage-dependent calcium channels (13, 38), which were not studied here.

In summary, these studies visualized the propagation of a TGF calcium wave from the MD area upstream toward proximal segments of the AA as well as toward intraglomerular elements. In light of the findings, not only the extraglomerular mesangium and the AA but all cells of the glomerulus play integral and active roles in a functional syncytium that regulates glomerular filtration during TGF. Another important function of the calcium wave is to coordinate the function of adjacent nephrons through glomeruli that belong to the same interlobular artery. Extracellular ATP is the key mediator of the TGF calcium wave, but subsequent degradation of ATP to adenosine may help maintain TGF-induced vasoconstrictions according to the current literature.

GRANTS

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