Phospholemman expression in extraglomerular mesangium and afferent arteriole of the juxtaglomerular apparatus

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Wetzel, Randall K., and Kathleen J. Sweadner. Phospholemman expression in extraglomerular mesangium and afferent arteriole of the juxtaglomerular apparatus. Am J Physiol Renal Physiol 285: F121–F129, 2003.—The molecular mechanisms with which the juxtaglomerular apparatus accomplishes its twin functions, acute regulation of glomerular blood flow and secretion of renin, are still not clearly understood. Least understood is the role of the extraglomerular mesangial (EM) cells, also known as laciis or Goormaghtigh cells, which lie sandwiched between the macula densa and the afferent and efferent arterioles. Here, we report that immunoreactivity for phospholemman (FXYD1), a single-span membrane protein homologous to the gamma (γ)2-subunit of the Na,K-ATPase, is found in the kidney in EM cells with the Na,K-ATPase β2-subunit and in cortical blood vessels and the afferent arteriole with Na,K-ATPase α2 and β2. Phospholemman’s distribution in EM cells is distinct from that of the Na,K-ATPase γ-subunit, which is found on the basolateral surface of macula densa cells with Na,K-ATPase α1 and β1. Phospholemman is a major kinase target, and its location in the juxtaglomerular apparatus suggests that it is involved in tubuloglomerular feedback.

The renal juxtaglomerular apparatus (JGA) is a specialized contact region between the glomerulus and cortical thick ascending limb (CTAL) that plays an active role in renal ion homeostasis. The JGA senses changes in the ionic composition of fluid in the lumen of the CTAL and modulates vascular tone and renin secretion in the arterioles. The JGA is composed of three specialized cell types, macula densa cells, extraglomerular mesangial (EM) cells, and granular cells in the arteriolar wall. The macula densa cells, a patch of specialized cells in the CTAL, express Na+/K+-2Cl− cotransporter on their apical (luminal) surface and Na,K-ATPase and chloride channels on their basolateral surface. These and other carriers facilitate the transport of NaCl from the lumen of the CTAL across the apical membrane and across the basolateral membrane to the interstitial cleft (for review, see Ref. 39). Adjacent to the interstitial cleft, and lying between the macula densa cells and arterioles, is a pyramidal patch of EM cells. These cells are linked to each other and to granular cells by gap junctions. EM cells also contain myofilaments and may be contractile. Granular cells, also known as juxtaglomerular cells, are modified smooth muscle cells of the afferent arteriole. These cells produce renin, which is stored in cytoplasmic granules and released into the blood at a rate that is increased by a decrease in the concentration of NaCl in the lumen of the CTAL (39). An increase in luminal NaCl produces swelling of macula densa cells and rapid constriction of the proximal and intraglomerular afferent arteriole (36).

Phospholemman (PLM) is a 72-amino acid single-span membrane protein that belongs to the FXYD gene family, along with the Na,K-ATPase γ2-subunit (34, 45). PLM was initially discovered as a readily phosphorylated component of skeletal and cardiac sarcolemma sensitive to adrenergic agents and insulin. It is phosphorylated by PKA, PKC, NIMA kinase, and myotonic dystrophy protein kinase (31, 32, 49). PLM induces chloride and sodium conductances and increases taurine flux in oocytes, lipid bilayers, and cultured astrocytes (summarized in Ref. 9), but it is possible that this is not its primary physiological function. PLM overexpression in cardiac myocytes alters Ca2+ transients and contractility (43).

A more recent hypothesis is that similar to its FXYD homologs, the γ-subunit of the Na,K-ATPase (FXYD2), CHIF (FXYD4), and the dogfish shark salt gland protein PLMS, PLM associates with the Na,K-ATPase as an accessory subunit to alter transport properties. When expressed with Na,K-ATPase α- and β-subunits, the γ-subunit has the net effect of decreasing the pump’s apparent sodium affinity (1, 4, 7, 37, 38), whereas CHIF does the opposite, increasing apparent sodium affinity (7, 17). Phosphorylation of PLMS is proposed to cause its dissociation from Na,K-ATPase and activation of the enzyme (25). These observations suggest that FXYD proteins may be specialized for different specific alterations of sodium pump function. Direct evidence has recently been found that PLM reduces the affinity of the Na,K-ATPase for Na,K-ATPase in oocytes, just as γ does (13), and we observed the common precipitation and copurification of PLM.
with Na,K-ATPase in preparations from brain and an inhibitory effect of blocking antibodies (16).

Small amounts of PLM mRNA have been detected in mouse and human kidney (9, 12), leading to the hypothesis that PLM might be expressed selectively in particular structures: either in blood vessels to regulate smooth muscle contractility or in a nephron segment to regulate ion transport. The other FXYD proteins in the kidney, γ and CHIF, have nonoverlapping segment distributions (17), and some structures such as CTAL have neither protein (42, 51). Here, we report the coexpression of immunoreactivity for PLM with identified subunits of the Na,K-ATPase in the kidney exclusively in EM cells, granular cells, and renal arterioles, in a distribution distinct from that of γ in the macula densa.

MATERIALS AND METHODS

Tissue preparation. All animals (6- to 7-wk-old CD rats) were given food and water ad libitum for 5 or more days, maintained on a constant light-dark cycle, and killed midmorning. Ether-anesthetized rats were fixed by transcardiac perfusion with 2% paraformaldehyde in periodate-lysine buffer and kidneys were sectioned on a cryostat as described in detail earlier (51).

Antibodies. Antibodies used included the following. PLM-C1 is a rabbit polyclonal antibody raised against a peptide representing the COOH terminus of canine PLM (30) (gift of Dr. L. R. Jones, Kranert Institute of Cardiology, Indianapolis, IN). The antibody has been particularly well characterized, including the use of PepSpot overlapping synthetic peptide technology, with which the minimum epitope was determined to be the sequence FRSSIRRL, which is a subset of the immunizing peptide GTFRSSIRRLSTRRR (16). It specifically immunoprecipitates PLM. This antibody also appropriately stains the sarcolemma of cardiac and skeletal muscle (data not shown), and its use to detect and characterize PLM in the nervous system can be seen in Feschenko et al. (16). In control experiments, we demonstrated that preincubation of the antibody with the immunizing peptide blocked its ability to bind to PLM (Arystarkhova E and Sweedner KJ, unpublished observations).

McK1 and McB2 are mouse monoclonal antibodies specific for the Na,K-ATPase α1- and α2-subunits, respectively (2). To detect Na,K-ATPase α2s, we used XVI-F9G10 (Affinity Bioreagents), and to detect β2s, we used RNT-β2s (3). GP50 is a mouse monoclonal antibody specific for the Na,K-ATPase β2-subunit (6) (gift of Dr. J. Gurd, Univ. of Toronto, and Dr. P. W. Beesley, Royal Holloway and Bedford New College, Egham, UK). The recent use of all of the above Na,K-ATPase antibodies in the retina and ciliary body can be seen in Ref. 50. α5s Is a monoclonal antibody that recognizes all of the isoforms of the Na,K-ATPase (Developmental Studies Hy-bridoma Bank, Univ. of Iowa). Monoclonal antibody McG-11H is specific for both splice variants of the Na,K-ATPase γ-subunit (51). Anti-neuronal nitric oxide synthase (nNOS), a known marker of macula densa cells (46), is a polyclonal antibody from Zymed (San Francisco, CA). Anti-renin polyclonal antibody from Zymed (San Francisco, CA). Anti-renin polyclonal antibody from Zymed (San Francisco, CA). Anti-neuronal nitric oxide synthase (nNOS), a known marker of macula densa cells (46), is a polyclonal antibody from Zymed (San Francisco, CA). Anti-neuronal nitric oxide synthase (nNOS), a known marker of macula densa cells (46), is a polyclonal antibody from Zymed (San Francisco, CA). Anti-neuronal nitric oxide synthase (nNOS), a known marker of macula densa cells (46), is a polyclonal antibody from Zymed (San Francisco, CA). Anti-neuronal nitric oxide synthase (nNOS), a known marker of macula densa cells (46), is a polyclonal antibody from Zymed (San Francisco, CA). Anti-neuronal nitric oxide synthase (nNOS), a known marker of macula densa cells (46), is a polyclonal antibody from Zymed (San Francisco, CA). Anti-neuronal nitric oxide synthase (nNOS), a known marker of macula densa cells (46), is a polyclonal antibody from Zymed (San Francisco, CA). Anti-neuronal nitric oxide synthase (nNOS), a known marker of macula densa cells (46), is a polyclonal antibody from Zymed (San Francisco, CA). Anti-neuronal nitric oxide synthase (nNOS), a known marker of macula densa cells (46), is a polyclonal antibody from Zymed (San Francisco, CA). Anti-neuronal nitric oxide synthase (nNOS), a known marker of macula densa cells (46), is a polyclonal antibody from Zymed (San Francisco, CA). Anti-neuronal nitric oxide synthase (nNOS), a known marker of macula densa cells (46), is a polyclonal antibody from Zymed (San Francisco, CA). Anti-neuronal nitric oxide synthase (nNOS), a known marker of macula densa cells (46), is a polyclonal antibody from Zymed (San Francisco, CA). Anti-neuronal nitric oxide synthase (nNOS), a known marker of macula densa cells (46), is a polyclonal antibody from Zymed (San Francisco, CA). Anti-neuronal nitric oxide synthase (nNOS), a known marker of macula densa cells (46), is a polyclonal antibody from Zymed (San Francisco, CA). Anti-neuronal nitric oxide synthase (nNOS), a known marker of macula densa cells (46), is a polyclonal antibody from Zymed (San Francisco, CA). Anti-neuronal nitric oxide synthase (nNOS), a known marker of macula densa cells (46), is a polyclonal antibody from Zymed (San Francisco, CA). Anti-neuronal nitric oxide synthase (nNOS), a known marker of macula densa cells (46), is a polyclonal antibody from Zymed (San Francisco, CA).

Immuno-fluorescence. Sections were rinsed in PBS (0.01 M sodium phosphate, 0.15 M NaCl, pH 7.2) for 5 min, transferred to 95°C 10 mM sodium citrate (pH 6.0) in a Coplin jar standing in a boiling water bath, and incubated for 20 min for antigen retrieval. (This step was not essential, but it brightened the immunofluorescence.) The Coplin jar containing the slides was then removed from the bath and allowed to cool for 20 min. Slides were reequilibrated in several changes of room temperature PBS over 30 min. For all subsequent incubations, the slides were laid flat in a dark moist box. The sections were covered (~50 μl/slide) with 1% SDS in PBS for 5 min. The slides were rinsed thoroughly in PBS (3 times for 10 min) and then similarly incubated with 1% normal goat serum and 0.5% NEN blocking reagent (New England Nuclear, Boston, MA) in PBS with 0.3% Triton X-100 (PBS) for 1 h at room temperature. This blocking solution was removed with an aspirator, and primary antibodies were immediately applied as described below.

For conventional double-label immunofluorescence, sections were incubated overnight at 4°C with a mixture of one mouse antibody and one rabbit antibody at the appropriate dilution in PBSt. The dilutions were as follows: 1:500 PLM-C1; 1:4 McK1; 1:500 anti-nNOS; 1:3 GP-50; 1:4 McB2; 1:5,000 anti-renin; 1:500 α5s; and 1:500 McG-11H. The slides were rinsed in PBS (3 times for 10 min) and then incubated in a mixture of Cy3-conjugated goat anti-mouse IgG (1:300; Jackson ImmunoResearch, West Grove, PA) and FITC-conjugated goat anti-rabbit IgG (1:200; Jackson ImmunoResearch, West Grove, PA) in PBSt for 2 h. Finally, they were rinsed in PBS and placed under a coverslip in Vectashield fluorescence mounting medium (Vector Laboratories, Burlingame, CA). For all experiments involving more than one primary antibody, omission of any one of those primary antibodies removed only the specific immunoreactivity due to that antibody.

For triple-label immunofluorescence involving two different antibodies from one species on the same section, tyramide amplification was used for one of the antibodies. Antibodies were tested at a series of increasing dilutions until they were completely undetectable by standard fluorescence. As a secondary antibody, we used a mixture of one 50,000 anti-nNOS and anti-renin; 1:750 McK1. Even at this high dilution, these antibodies were still detectable using tyramide amplification. New slides were treated for antigen retrieval using hot citrate and SDS and blocked as described above, and they were then incubated with anti-nNOS, anti-renin, or McK1 at the high dilution. The slides were rinsed in PBS as described above, incubated in biotinylated horse anti-mouse IgG or biotinylated goat anti-rabbit IgG (1:100 or 1:1,000, respectively, Vector Laboratories) in PBSt for 2 h, rinsed in PBS, incubated in streptavidin-HRP (1:100, NEN) in PBSt, rinsed in PBS, and then incubated in Cy3-Tyramide Reagent (1:100) in NEN amplification diluent (NEN) for 5–7 min. The slides were then rinsed for 5 min and then incubated with a second rabbit antibody and a mouse antibody in PBSt overnight at 4°C. They were then rinsed in PBS and incubated in Cy5-conjugated goat anti-mouse IgG (1:300) and FITC-conjugated goat anti-rabbit IgG (1:200; Jackson) in PBSt for 2 h. The slides were then rinsed and placed under a coverslip as described above.

Finally, all slides were examined and images were collected on a Nikon TE300 fluorescence microscope equipped with a Bio-Rad MRC 1024 scanning laser confocal system, version 3.2, or on a Zeiss Axiovert 200M microscope equipped with a Zeiss LSM5 PASCAL scanning laser confocal system, version 3.0.

RESULTS

Confocal immunofluorescence microscopy was used to localize PLM protein expression in the normal rat kidney using a well-characterized polyclonal antibody. All results reported are typical of multiple experi-
ments. At low magnification, strong PLM immunoreactivity was seen in a crescent-shaped patch of cells at the vascular pole of glomeruli (Fig. 1A). PLM immunoreactivity was also seen neatly outlining cells of cortical blood vessels (Fig. 1B). In sections that were double-labeled with PLM and Na,K-ATPase α₁-subunit antibodies, PLM immunoreactivity was found, with a nonpolar distribution, on the plasma membrane of elongated presumptive EM cells, whereas α₁-immunoreactivity was seen on the basolateral surface of the adjacent macula densa cells and in surrounding proximal convoluted tubule and distal convoluted tubule (DCT) (Fig. 2). Macula densa cells were identified by the characteristic rounded shape of the cells in an otherwise normal CTAL structure and by position contacting the glomerulus. Bright α₁-immunoreactivity was present in the CTAL and other nephron segments in a pattern that was consistent with previous studies, but no α₁-immunoreactivity was seen in EM cells or any other part of the glomerulus. We observed that in every glomerulus with an identifiable JGA visible in the section, PLM immunoreactivity was always found in the EM cells. Glomerular arterioles cut in cross section were occasionally seen as black holes adjacent to labeled EM cells in PLM-stained sections (Fig. 3).

To further identify the cells showing immunoreactivity in the JGA, we performed triple-label immunofluorescence with antibodies directed against nNOS and renin, which are known markers of macula densa cells and afferent arterioles, respectively. Na,K-ATPase α₁ was found in the basolateral membrane of macula densa cells marked with nNOS antibody stain of the cytoplasm, but PLM immunoreactivity was only seen in adjacent EM cells (Fig. 4, A–D). Renin, on the other hand, was found in the cytoplasm of the granular cells of the afferent arteriole, and these cells also showed PLM immunoreactivity in the plasma membrane (Fig. 4, E–H). No renin immunoreactivity was seen in the PLM-labeled EM cells or in the α₁-labeled macula densa cells. Because we never observed a JGA with two PLM-labeled arterioles, it is possible that PLM is only expressed in afferent arterioles (identified by the presence of renin) and not in efferent arterioles.

Because PLM did not appear to colocalize with the α₁-subunit of Na,K-ATPase, we used other isoform-specific antibodies to investigate the possibility that another sodium pump isoform was found in EM cells and blood vessels. The kidney was not detectably stained by well-characterized α₂- and β₂-antibodies (data not shown), although the antibodies work well on other tissues with the same protocol (26, 50). Immunoreactivity for α₂ and β₂ was seen, however. β₂-Immunoreactivity consistently colocalized with PLM throughout the cortex, in afferent arterioles and in EM cells (Fig. 5). As with PLM, no β₂-immunoreactivity
was seen in the glomerulus itself, in macula densa cells, or in any tubular segment of the nephron. Immunoreactivity for $\alpha_2$ colocalized with PLM in cortical blood vessels, but $\alpha_2$-immunoreactivity was not seen in EM cells (Fig. 6D). Even tyramide amplification of the $\alpha_2$-immunoreactivity was unable to produce visible stain in the EM cells (Fig. 6E). $\alpha_2$ Was also not detected in renin-reactive cells of the afferent arteriole, although the adjacent afferent arteriole was stained (Fig. 6G). Figure 6B shows an arteriole (paired arrows) that was PLM positive but $\alpha_2$ negative, not far from a clearly $\alpha_2$-positive blood vessel. Whether this arteriole was afferent or efferent is uncertain because no independent marker was used in this experiment. In sum, most detected blood vessel elements in the cortex did have immunoreactivity for $\alpha_2$, $\beta_2$, and PLM, but there were arteriolar segments without detectable $\alpha_2$. It is not possible to make a categorical statement that there is a difference between afferent and efferent arteriole on the basis of the available data; an efferent arteriole marker would be needed.

We used a pan-specific Na,K-ATPase $\alpha$-subunit antibody (“$\alpha_5$”) that is known to recognize $\alpha_1$, $\alpha_2$, and $\alpha_3$. Although clear $\alpha$-immunoreactivity was seen in nephron segments and macula densa cells, no $\alpha$ was detected in EM cells, even in overexposed micrographs (Fig. 7). It is possible, however, that the amount of $\alpha$ is simply below a detection threshold.

We and others previously reported that the Na,K-ATPase $\gamma$-subunit FXYD2 is expressed in macula densa as well as in other portions of the nephron, colocalizing with Na,K-ATPase $\alpha_1$ (37, 51). Figure 8 shows that $\gamma$-immunoreactivity was present in macula densa cells identified by cytoplasmic immunoreactivity for nNOS, but no $\gamma$-immunoreactivity was seen in the adjacent EM cells.

Figure 9 is a summary diagram of PLM and Na,K-ATPase immunoreactivity in the JGA. PLM and $\beta_2$-immunoreactivity in EM cells, granular cells, and arterioles are represented by green; $\gamma$ on the basolateral membrane of the macula densa cells is represented by red; and $\alpha_1$ on the basolateral membranes of macula densa and CTAL cells is represented by blue. Where red and blue immunoreactivity overlap in the macula densa basolateral membrane, the color is purple. $\alpha_2$-Immunoreactivity was seen in blood vessels, including arterioles.
the regular smooth muscle cells of the afferent arteriole (*).

**DISCUSSION**

**PLM and Na,K-ATPase expression in the JGA.** The distribution of PLM in the kidney was investigated because of the likelihood that it is a target of signaling pathways. Because PLM has been shown to associate with and regulate Na,K-ATPase (13, 16), we also attempted to determine whether it could be detected colocalized with Na,K-ATPase subunit isoforms. PLM immunoreactivity was found in the EM and granular cells of the JGA, in cortical blood vessels, and in afferent arterioles identified by continuity with renin-stained cells. In nearby structures, CTAL expressed no detectable FXYD protein, and macula densa expressed only γ. PLM immunoreactivity consistently colocalized with the Na,K-ATPase β2-subunit, but it was seen with α2 only in cortical blood vessels and afferent arterioles.

Fig. 5. Kidney section triple-labeled with nNOS (red), PLM (green), and Na,K-ATPase β2-antibodies (blue). A–D: low magnification showing an arteriole; E–H: high magnification of EM cells and the adjacent MD. PLM and β2 were colocalized in EM cells and in arterioles. No β2-immunoreactivity was seen in MD cells.

Fig. 6. Kidney section labeled with Na,K-ATPase α1 (red; A), PLM (green; B, C, and F), and Na,K-ATPase α2-antibodies (blue B and D; red E). B: merged images of A, C, and D. Arrows indicate an α2-negative, PLM-positive arteriole. α2-Immunoreactivity was colocalized with PLM in BV, but EM cells (arrowheads) were not stained by the α2-antibody. Tyramide amplification of α2-immunoreactivity did not produce visible immunoreactivity in EM cells (E) that were stained with PLM (F), shown here at higher magnification than in A–D. Arrows indicate the location of EM cells. G: afferent arteriole labeled for renin (green) and α2 (red).
In principle, it is possible that the PLM immunoreactivity is due to cross-reactivity with another protein, but this antibody has been extensively characterized and is competed by peptide. A very small amount of PLM has been detected on Western blot analysis of cortical tubule preparations, consistent with the presence of contaminating PLM-containing structures (Arystarkhova E and Sweadner KJ, unpublished observations). Another formal caveat is that the epitope of the antibody contains the phosphorylation sites, and it is possible in principle that PLM is present in other renal segments, but its immunoreactivity is masked by being in a phosphorylated state.

No Na,K-ATPase α-isoform was detected in EM cells or in renin-positive granular cells, and as reported by others (20), none was detected in the glomerulus itself. It is notable that although the afferent arteriole enters and branches in the glomerulus, where the lumen collapses in response to tubular NaCl (36), immunoreactivity for PLM, α₂, or β₂ was not observed in the intraglomerular vascular structure.

PLM is the third FXYD protein to be detected in the kidney. The gene family is named for a sequence motif (Pro-Phe-X-Tyr-Asp) at the beginning of a 35-amino acid-conserved domain (45). The Na,K-ATPase γ-subunit (FXYD2) is expressed at levels parallel to γ₁ in most segments, with segment specificity in the distribution of its splice variants, γ₁ and γ₂, in proximal tubule, distal tubule, and thick ascending limb in the outer stripe of the outer medulla (37, 51). No γ was detected in the CTAL and collecting duct (5, 51), whereas CHIF expression was confined to collecting duct (17, 42). In this study, PLM immunoreactivity was restricted to blood vessels and EM cells. The three FXYD proteins thus have no detected overlap in their distribution in the kidney.

It has been a matter of some controversy which isoforms of the Na,K-ATPase are expressed in the kidney, although there is now consensus that there is very little detectable expression of α₂ or α₃ (15, 24). All tubular segments of the nephron express Na,K-ATPase α₁- and β₁-subunits on their basolateral surface, although at different levels as measured by activity (21, 28), Western blot analysis (29), or immunocytochemistry (20, 51). The highest Na,K-ATPase expression is in

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**Fig. 7.** Kidney cortex labeled with a pan-specific Na,K-ATPase α-subunit antibody. Labeled MD cells were clearly seen with normal exposures (arrowhead). Right: image was overexposed to show that there was no detectable α-immunoreactivity in the EM cells (arrow).

**Fig. 8.** Kidney section labeled with nNOS (red), PLM (green), and Na,K-ATPase γ-subunit (blue) antibodies. D: merged images of A–C. γ-Immunoreactivity was colocalized with nNOS in MD cells but not in EM cells. The arrow indicates the position of the interstitial cleft.

**Fig. 9.** Diagram of PLM and Na,K-ATPase immunoreactivity in the juxtaglomerular apparatus. Green indicates PLM and β₂-immunoreactivity in arterioles, granular cells, and EM cells; red indicates γ₁-subunit immunoreactivity on the basolateral surface of MD cells; and blue indicates α₁-subunit immunoreactivity on the basolateral surface of MD and CTAL cells. Afferent arteriolar smooth muscle cells also express α₂ adjacent to the renin-positive cells (*)

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the thick ascending limb, DCT, and connecting tubule. The search for other isoforms was motivated initially by observations of sodium affinity differences between segments, but these differences can now be explained, at least in part, by segment-specific expression of the modulators γ and CHIF. To our knowledge, α2 has not been detected as a protein in the kidney before, although β2 was reported as a predominant β-form in fetal human kidney (11). We could not confirm a report of the presence of β2 in several nephron segments (47). The detection of α2 and β2 here in minor structures reflects advances in available methodology: antigen retrieval from fixed tissue sections and confocal microscopy.

It has recently been proposed that macula densa cells in the rabbit lack Na,K-ATPase and recycle sodium at the luminal membrane by the use of the nongastric X,K-ATPase and other transporters instead (35). The conclusion that Na,K-ATPase was absent was based on the absence of detectable immunoreactivity in macula densa for Na,K-ATPase by antibody 6F, which is specific for α1 (2). Here, Na,K-ATPase α1- and γ-subunits colocalized on the basolateral surface of macula densa cells as we and others previously reported (37, 51) and were not detected in any other JGA component. In the rat, we also observed clear 6F immunoreactivity of macula densa like that shown here with the McK1 and α5 antibodies (data not shown). Because staining by 6F has been shown to benefit from antigen retrieval methods (10), it is possible that immunoreactivity for α1 might have been below the limit of detection in the rabbit. There are other physiological differences between rat and rabbit kidney, however, and the rabbit may differ from the rat in this respect.

Because there is always a limit to antibody sensitivity, we are equally cautious about interpreting the apparent lack of any Na,K-ATPase α-subunit observed here in glomerulus and EM cells, even with tyramide amplification and an isoform pan-specific antibody (α5). It seems unlikely that the EM cells are devoid of a Na,K-ATPase α-subunit, because β2 is not known to occur elsewhere without an α-subunit, and all mammalian cells are thought to express some Na,K-ATPase. Cultured glomerular mesangial cells have been shown to express α1- and β1-mRNA but not α2 or α3 (33). It is theoretically possible that an H,K-ATPase or X,K-ATPase α-subunit is paired with the Na,K-ATPase β2-subunit; however, we stained kidney sections with antibodies specific to either the colonic or gastric H,K-ATPase and no glomerular or juxtaglomerular expression was seen (Wetzel and Sweadner, unpublished observations). It is also possible that α4, a Na,K-ATPase found so far only in sperm (41), is present, but it seems more likely that Na,K-ATPase α-subunit expression is just below the threshold of detection or the epitope is somehow masked. The prominent immunoreactivity for PLM does not necessarily mean that PLM is present in EM cells without Na,K-ATPase, because the polyclonal PLM antibody used is very strong and may simply be more sensitive or have a lower threshold than any of the monoclonal α-antibodies used. The low level of α does suggest, however, that sodium and potassium transport is not as quantitatively important a function of EM cells as in nephron tubules.

**Implications for the JGA.** It is well-established that changes in luminal NaCl in the CTAL affect vascular tone in the glomerulus through tubuloglomerular feedback. There are two mechanisms for this effect, a short-term local regulation of afferent arteriole resistance and a long-term systemic regulation of efferent arteriole resistance that involves the release of renin from the granular cells of the afferent arteriole and subsequent activation of the renin-angiotensin system (for review, see Ref. 39). The initial trigger for both is NaCl uptake by macula densa cells. Although EM and granular cells are linked by gap junctions, they are physically separated from the macula densa cells by the interstitial cleft, so an extracellular messenger is required to pass the signal from the macula densa cells to the afferent arteriole. Several messengers have been proposed, including adenosine, prostaglandins, and nitric oxide (for review, see Ref. 18). Although these messengers are clearly involved in the regulation of vascular tone, it is also likely that NaCl transported across the macula densa cells has an effect on EM cells. Matsunaga et al. (27) reviewed evidence that mesangial cell functions are modulated by changes in extracellular chloride. EM cells have been shown to express the Na +–K +–2Cl – cotransporter BSC2 (19), and interestingly, BSC2 has a distribution in the JGA that is apparently identical to that of PLM and β2: only in EM cells and afferent arterioles. Chloride regulates stimulated calcium influx in mesangial cells (22, 44). Calcium influx and mobilization may be the signal that passes to granular cells and afferent arteriole smooth muscle cells (via gap junctions), affecting vascular contractility in the afferent arteriole and decreasing renin release from granular cells (14, 40). Calcium is mobilized from intracellular stores and from influx via the Na +/Ca 2+ exchanger NCX1 in EM cells as well as arterioles (8).

The functional significance of PLM expression in EM cells and the granular and smooth muscle cells of the afferent arteriole is not known, but it is attractive to speculate that PLM modulates the movements of ions, thereby participating in tubuloglomerular feedback. Because PLM is a kinase target with two distinct phosphorylation sites, it may be phosphorylated and modified by one or more diffusible signaling molecules through receptors. It is possible that PLM activates a chloride channel in EM cells as it does in Xenopus laevis oocytes (30). Its relationship to other FXYD proteins and coprecipitation with Na,K-ATPase, however, suggest that PLM may modulate Na,K-ATPase activity. Through that, it may affect intracellular calcium levels through Na +/Ca 2+ exchange. Overexpression of PLM in cardiac myocytes has been shown to affect contractility as a complex function of Ca 2+ concentration (43). It has been suggested that mesangial cell contractility functions to counteract glomerular distension caused by increased perfusion rates (23),
and PLM may play a role in regulating that contractility in EM cells. However, PLM has also been implicated in a very different phenomenon, insulin-dependent Glut4 translocation in adipocytes (48). Consequently, it would be premature to predict its precise molecular role in the JGA. It does, however, provide a readily studied candidate target for tubuloglomerular feedback pathways.

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