Real-time imaging of renin release in vitro

János Peti-Peterdi,1,2 Atilia Fintha,1 Amanda L. Fuson,1 Albert Tousson,3 and Robert H. Chow.1

1Nephrology Research and Training Center, Division of Nephrology, Department of Medicine, University of Alabama at Birmingham, Birmingham, Alabama 35294; and 3Department of Physiology and Biophysics and Zilkha Neurogenetic Institute, Keck School of Medicine, University of Southern California, Los Angeles, California 90089

Submitted 2 December 2003; accepted in final form 6 April 2004

Address for reprint requests and other correspondence: J. Peti-Peterdi, ZNI335, MC 2821, 1501 San Pablo St., Los Angeles, CA 90089-2821 (E-mail: petipete@usc.edu).

Renin release has been extensively studied using electron microscopy (17, 26, 29), radioimmunoassays (2, 3, 12, 22), and patch-clamp techniques (8). Fluorescence imaging with quinacrine was used to identify the renin-containing part of the afferent arteriole (5) and to study the effects of hemorrhage and ischemia on renal renin content (1). Most of the available renin assays work with cumbersome and potentially inaccurate techniques, and none of the existing experimental methods allow direct visualization of renin release using time-lapse imaging in a living system. Exocytosis has been suggested as the main mechanism of renin release (8, 16, 22, 26); however, to date, this process has not been visualized.

Recently, we applied multiphoton excitation laser-scanning fluorescence microscopy to visualize the microperfused JGA (20). This imaging technique allowed us to study the function of this poorly accessible, complex cell structure in vitro, with high optical resolution. The same experimental approach was used in the present work to visualize juxtaglomerular granular cells, individual renin granules, the morphology and dynamics of renin release, and tissue renin activity using acidotropic fluorophores.

MATERIALS AND METHODS

Salt diet. Separate groups of New Zealand White rabbits (0.5–1.0 kg, Myrtle) were fed standard (0.3% NaCl, 8630 Harlan Teklad, Madison, WI) or low-salt (0.01% NaCl, TD 90188) rabbit chow for a minimum of 1 wk.

All animals procedures and protocols were approved by the Institutional Animal Care and Use Committee of the University of Alabama at Birmingham.

Imaging renin granules and release. Afferent arterioles with attached glomeruli preparations were hand-dissected from rabbit kidneys and microperfused with 150 mM NaCl containing Ringer solution as previously described (18–20). Individual preparations were transferred to a thermoregulated Lucite chamber mounted on a Leica DM IRBE inverted microscope and visualized using a ×63 oilimmersion lens. In some experiments, the fluorescent membrane staining dye 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene p-toluenesulfonate (TMA-DPH; 1 μM, Molecular Probes, Eugene, OR) was continuously present in the arteriolar perfusate to visualize cellular structures. Acidotropic fluorophores, e.g., quinacrine (Sigma, St. Louis, MO) and LysoTracker-Red (Molecular Probes), that freely pass through cell membranes and accumulate in acidic cellular compartments, were used to selectively label renin granules.

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
Quinacrine (25 μM) was administered to the arteriolar perfusate for up to 5 min and then washed out. For visualization of fluorescence, TMA-DPH (755 nm) or quinacrine (800 nm) was excited using a two-photon laser composed of a photo-diode pump laser (5 W, Verdi) and a mode-locked titanium-sapphire laser (Mira, both from Coherent Laser Group, Santa Clara, CA) in a Leica TCS SP confocal imaging system (Leica Microsystems, Heidelberg, Germany) equipped with an acousto-optical tunable filter (AOTF). Fluorescence emission was detected at a peak of 430 (TMA-DPH) or 510 nm (quinacrine). Images were collected and analyzed with the Leica LCS imaging software in time (xyt) series, at a rate of 1 frame/10 s, or in some experiments, 1 frame/0.8 s. Renin release was stimulated by adding 100 μM isoprote- terol (Sigma) to the bathing Ringer solution or, in some experiments, by reducing arteriolar perfusion pressure to zero (stop flow). Renin release was also studied in cultured, living As4.1 cells prepared according to the supplier’s instructions (ATCC, Manassas, VA). As4.1 cells are derived from a renin-expressing kidney tumor induced by tissue-specific oncogene-mediated tumorigenesis in transgenic mice, and these cells have been shown to express high levels of renin mRNA and synthesize prorenin and renin (27).

Imaging renin activity. In some experiments, renin granules were labeled after arteriolar arteries were loaded with LysoTracker-Red (5 μM) for 5 min. LysoTracker-Red was excited at 568 nm using a Kr ion laser, and fluorescence emission was detected at a peak of 590 nm. Simultaneously, the 5-(2-aminoethylamino)naphthalene-1-sulfonic acid (EDANS)- and DABCYL-conjugated fluorescent renin substrate (DABCYL-γ-Abu-Ile-His-Pro-Phe-His-Leu-Val-Ile-His-Thr, AnaSpec, San Jose, CA) was added to the bathing solution (2 μM). In the absence of renin activity, EDANS fluorescence is quenched by the acceptor molecule DABCYL due to fluorescence resonance energy transfer (FRET). However, when cleaved by renin, the two fluorophores separate, giving rise to bright EDANS fluorescence. The EDANS- and DABCYL-conjugated fluorescent renin substrate has been developed and validated earlier (11). EDANS fluorescence emission was detected at a peak of 490 nm in response to two-photon excitation at 720 nm. Sequential scanning of LysoTracker-Red and EDANS fluorescence was performed using Leica LCS imaging software, as described above.

Immunohistochemistry. Mouse kidneys were perfusion-fixed with paraformaldehyde, and tissue sections were processed as described earlier (19). Sections were subjected to microwave antigen retrieval (for 10 min) before staining and blocked for 40 min with PBS-Tween containing 2% goat serum to lower background fluorescence. After subsequent washings in PBS, tissues were treated with a rabbit polyclonal renin antibody (m752, SLU-1017, 1:100, purchased from Dr. E. J. Brandt, Technology Transfer Of- fice, St. Louis University, St. Louis, MO). After washing, this was followed by a 40-min incubation with Alexa 594-conjugated donkey anti-rabbit IgG (1:2,500, Molecular Probes). Sections were then counterstained with quinacrine (25 μM) for 20 min and then washed twice. Some sections were counterstained with LysoTracker-Red (Alexa 488-conjugated secondary antibody was used in these experiments) and others with quinacrine and LysoTracker-Red simultaneously. Sections were mounted with Vectashield media, containing 4,6-diamino-2-phenylindole for nuclear staining (Vector Labs). Tissue sections were examined with an Olympus IX70 inverted epifluorescence microscope using a UApo 340×40 objective. 4,6-Diamino-2-phenylindole was excited at 357 nm, quinacrine at 403 nm, and Alexa 488 at 493 nm, and Alexa 594 and LysoTracker-Red at 572 nm using excitation filters (Chroma Technology, Rockingham, VT). Images were captured using a SenSys digital camera and IPLab Spectrum software equipped with a power microtome (Signal Analytics).

RESULTS

Imaging renin granules. Perfusion loading the afferent arte- rirole-glomerulus complex with the acidotropic fluorophore quinacrine selectively labeled intracellular, granular structures in select cells of the terminal, juxtaglomerular portion of afferent arterioles (Fig. 1). Labeling was very intense, clearly localized in spherical subcellular compartments, and lasted for many hours even after quinacrine was washed out of the perfusate. These structures were identified as renin granules in juxtaglomerular granular cells; renin immunohistochemistry with subsequent quinacrine staining colocalized quinacrine fluorescence with renin labeling (Fig. 2A). It should be noted, however, that the overlap appears to be incomplete, and there is additional cytosolic renin labeling that is not stained by quinacrine; this may be an artifact due to aldehyde fixation and/or sectioning techniques used with immunohistochemistry. Similar results were obtained when renin granules were stained for renin immunohistochemistry and with the lysosome marker LysoTracker-Red (data not shown). Quinacrine and LysoTracker-Red dyes label the same granular structures in the afferent arteriole and diffusely distributed lysosomes in the proximal tubule, as confirmed by their overlapping signal (Fig. 2C).

Figure 3 demonstrates that quinacrine was also useful in tracking physiological changes in renin content. Although renin content appeared to be highly variable, there were an average of 4.1 ± 0.3 granular cells and 67.4 ± 16.5 renin granules/focal plane with normal-salt intake (n = 8). A low-salt diet for 1 wk caused a tremendous increase in both the number of individual granules (214 ± 13.8) and in the number of granular cells (10.7 ± 0.7) per focal plane in the afferent arteriole (n = 15). The size of individual granules was highly variable; we observed several large granules of 2–3 μm in diameter.

![Fig. 1. Visualization of quinacrine fluorescence (green) in the in vitro microperfused afferent arteriole. Tissue was costained with the membrane marker 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene p-toluenesulfonate (TMA-DPH; blue) to enable visualization of the anatomic structure. Quinacrine fluorescence (renin granules) was localized to select cells in the terminal afferent arteriole (AA). G, glomerulus; MD, macula densa. Bar = 20 μm.](http://ajprenal.physiology.org/Downloadedfrom/10.22033.2.onJune17,2017)
Imaging renin release. We used time-lapse imaging in 23 quinacrine-loaded afferent arterioles, initially at a rate of 1 frame/10 s for 10 min, to study the dynamics and morphology of renin release. In control experiments (not shown), the number of individual granules per focal plane appeared to be constant over time; in some preparations, very rarely we observed the disappearance of one granule. The addition of the classic β-agonist isoproterenol, a well-characterized method of stimulating renin release, to the bathing solution (100 μM) with normal-salt intake caused the dimming and disappearance of quinacrine fluorescence from four to five individual renin granules/focal plane over the course of 10 min. Isoproterenol caused a major renin release in 15 low-salt preparations that we observed (Fig. 4). On average, 40 ± 4.2% of granules disappeared in 600 s with no detectable movement relative to the cell membrane (a complete digital video of the same preparation is available in a supplementary file at http://ajprenal.physiology.org/cgi/content/full/00420.2003/DC1). The disappearance of quinacrine fluorescence (renin release) was specific for isoproterenol, because bath superfusion with vehicle only caused no significant change in the number of granules, similar to control experiments (Fig. 4B). Release appeared to be into both the luminal and interstitial compartments of the JGA. Also, there appeared to be a quantal release of granular contents; i.e., an all-or-none phenomenon. Similar to isoproterenol, reductions in arteriolar perfusion pressure, another key stimulus for renin release, caused significant renin degranulation, visualized as a reduction in quinacrine fluorescence intensity (Fig. 4C).

Morphology of renin release, on the subcellular level, can be studied with high resolution, as demonstrated in Fig. 5 (a complete digital video of the same preparation is available in a supplementary file at the URL noted above). Even a higher rate of imaging (1 image/0.8 s) provided no signs of granule trafficking, any movement, or change in size during release, only the dimming and disappearance of quinacrine fluorescence. Those individual renin granules destined for release disappeared in <0.8 s in response to 100 μM isoproterenol, probably due to a fast dilution of granular content (quinacrine) in the bath fluid. Indeed, simultaneously with the disappearance of granules, a steady increase in quinacrine fluorescence intensity was detected in the bath fluid around granular cells (Fig. 4C).
Imaging renin activity. Next, we studied whether there is detectable renin activity around granular cells of the perfused afferent arteriole that parallels the observed degranulation. Because of an almost complete overlap in both the excitation and emission spectra of quinacrine and the fluorophore used in the renin substrate (EDANS), these experiments utilized LysoTracker-Red, another acidotropic fluorescent dye. Figure 7 illustrates that, similar to quinacrine, LysoTracker-Red selectively stained renin granules in the afferent arteriole. Furthermore, a $2.6 \pm 0.7$-fold increase in EDANS fluorescence and a $23.3 \pm 4.8\%$ reduction in LysoTracker-Red fluorescence ($n = 5$) were observed in response to the addition of isoproterenol, indicating renin activity. A representative recording (Fig. 7C) demonstrates that the time course of an increase in EDANS fluorescence (renin activity) and the simultaneous decrease in LysoTracker-Red fluorescence (renin release) were similar.

**DISCUSSION**

This technical report provides, for the first time, direct visualization of renin degranulation in a living system in real time. Instead of tagging renin with fluorescent proteins (13), we found that acidotropic fluorophores, such as quinacrine and LysoTrackers, are extremely useful in easily and selectively labeling renin granules in both living and fixed tissues. These fluorophores are amine group-containing weak bases that are freely permeant to cell membranes and selectively accumulate in cellular compartments with low internal pH. For this reason, they can be used for labeling and tracing acidic organelles, such as renin granules (pH is $\sim 5.5$) in living juxtaglomerular cells. Indeed, quinacrine has been shown before to selectively stain the intracellular storage granules in renin-producing juxtaglomerular cells in the terminal afferent arteriole (1, 5). Quinacrine has been used to visualize phagosomes (6) and secretory vesicle release in many other organs and systems (4, 25).

In the present study, quinacrine and LysoTracker selectively labeled renin granules in juxtaglomerular granular cells in the microperfused afferent arteriole as well as in As4.1 cells, a granular cell line. Localization of quinacrine fluorescence to renin granules was based on anatomic features; i.e., fluorescence was visible only in select cells of the terminal, juxtaglomerular afferent arteriole (Fig. 1) in spherically shaped granules (Figs. 1–7). In addition, renin immunohistochemistry with subsequent quinacrine counterstaining colocalized renin granules and quinacrine fluorescence (Fig. 2). Quinacrine-labeled renin granules can be easily distinguished from the much smaller, diffusely distributed lysosomes, and the large cell nuclei that are, although to a much smaller extent, also stained with this fluorophore (Figs. 2–5). High renin states, such as a low-salt diet, can be diagnosed based on quinacrine fluorescence intensity (Fig. 3), the number of granular cells, and individual granules. It should be mentioned, however, that $\sim 25$–$50\%$ of the in vivo renin content of the microdissected JGA is lost due to the isolation procedure (9, 22). Therefore, we standardized and kept the duration of isolation to a minimum, and dissection was always performed at 4°C to prevent degranulation. Also, quinacrine has long been used for antimalarial, antirheumatic, contraceptive, nuclear staining, antioxidant, etc. purposes (28), and it also interacts, in high doses, with many intracellular signaling pathways, enzymes, and

Additional work utilized As4.1 cells, a granular cell line, to further study renin release using quinacrine. As with native tissue, quinacrine selectively labeled renin granules in As4.1 cells (Fig. 6); however, these cells appeared to release renin spontaneously. In fact, a variety of stimuli caused no effect on quinacrine fluorescence intensity (renin content), including isoproterenol, hypotonic challenge, and calcium removal (not shown). Nonetheless, during spontaneous renin release, we observed classic signs of granule exocytosis, the emptying of granule content (Fig. 6A) associated with an extracellular flash of quinacrine fluorescence (Fig. 6B).
receptors, including phospholipase A₂, NADH, and NADPH (10). Low doses of quinacrine are, however, considered safe (28), and present studies used this drug temporarily, for only 1–2 min of loading; after that time it was washed out. There was absolutely no indication that quinacrine, by itself, alters the number or morphology of renin granules, or release, and labeling and imaging renin granules with LysoTracker-Red, another fluorophore, appeared to give the same results (Figs. 4 and 7).

Present studies support the previous observation in embedded kidney tissue after supravital staining (1) that quinacrine fluorescence in granular cells is released when renin is released. In response to the β-agonist isoproterenol, a classic stimulus for renin release (2–3, 12), we observed the dimming and disappearance, but no movement, of quinacrine-labeled renin granules. The disappearance was very rapid (<0.8 s) and discrete, probably caused by a fast dilution of the dye and not by changes in intragranular pH, because quinacrine and LysoTracker fluorescence are not sensitive to wide variations in pH (data not shown). Also supportive of quinacrine/renin release, after a stimulus, there was a steady increase in quinacrine fluorescence intensity in the bath fluid around granular cells (Fig. 4C). Changes in the focal plane due to possible movements were prevented by firmly holding the glomerulus-arte-

Fig. 5. Higher magnification of quinacrine-labeled renin granules (A, arrows) and their disappearance in response to isoproterenol (B) in tissue from rabbits on a low-salt diet. Bar = 10 μm. A digital video of same preparation, at a rate of 1 frame/0.8 s, is available in a supplementary file (see RESULTS).

Fig. 6. Classic signs of granule exocytosis observed in A54.1 cells stained with quinacrine. Spontaneous emptying of granule content (A, arrows) associated with an extracellular flash of quinacrine fluorescence (B, arrows) was observed.
riole complex with the help of a micropipette. There was no change in the position of cell nuclei or those renin granules not destined for release (see supplementary video file noted in RESULTS). The only exception occurred when a decrease in or cessation of arteriole perfusion was used as a release stimulus (Fig. 4C). Here, not the absolute number of granules but a reduction in quinacrine fluorescence intensity was analyzed as a marker of renin release, an approach that appeared to be equally useful.

The dynamics of granule disappearance are consistent with previous reports on the rate of renin release (22, 23). In control studies, we observed a discontinuous pattern of release, rarely one granule disappearing, an almost constant number of granules in cells (undisturbed release, Fig. 4B). Stimulation with isoproterenol caused four to five granules to disappear in 10 min in control tissue, but in contrast, a major renin release was observed in low-salt preparations. Several low-salt afferent arterioles released ~40% of their renin content in 10–15 min (Figs. 4 and 7, and supplementary video). This is an unusually enormous amount, probably due to the combined stimulatory effect of a low-salt diet and a high dose of isoproterenol used. Actually, it was our purpose to show clear, significant responses in this technical report that are easy to read. These data confirm that not only renin content but also the fraction of content released (readily releasable pool) are substantially higher in kidneys from salt-deprived animals (7).

The fast dimming and disappearance of quinacrine-labeled renin granules, the emptying of granule content, and the extracellular quinacrine cloud or flash are all classic signs of exocytosis, the main mechanism for renin release, as suggested by many investigators (8, 16, 22, 26). There was no indication of granule shrinkage or increasing quinacrine fluorescence in the cytosol, which argues against the cytosolic solubilization theory of renin release (14, 29). Absolutely no detectable movement of renin granules during release is consistent with earlier ultrastructural findings using electron microscopy (26) that a previous fusion between the cell and granule membranes exists and that the mature granules are ready and about to expel their content into the extracellular space. Previously described deep invaginations of the cellular membrane (17) might explain why and how deep-lying granules, even those close to the cell nucleus, release their content into the interstitium. Furthermore, these data are consistent with the episodic and quantal release of renin (22, 23), i.e., discharge of entire amounts of renin located in single, individual granules.

Also supportive of renin release from disappearing quinacrine-labeled granules is the finding that simultaneously with degranulation there was a detectable increase in renin activity of bath fluid around granular cells (Fig. 7). The visualization of renin activity using fluorescence microscopy is also a novelty in these studies and was performed using a newly developed (11) fluorogenic peptide substrate (analogous to assaying human immunodeficiency virus protease activity) containing a sequence of human angiotensinogen and two fluorophores. Proteolytic cleavage (renin activity) releases a fragment containing only the EDANS fluorophore, thus liberating it from the quenching effect (FRET) of the nearby DABCYL chromophore. Isoproterenol stimulation caused a steady increase in EDANS fluorescence around granular cells (Fig. 7). The majority of renin activity most likely originated from renin granules at the exposed outside of the vessel, because renin substrate was added to only the bath fluid and not to the afferent arteriole.
arteriole perfusate. This finding confirms previous in vivo data that juxtaglomerular granular cells secrete renin into the interstitium (15). Future studies, with renin substrate in both the arteriole lumen and bath, would allow quantification of the fraction of renin that is secreted into the vascular lumen or the interstitium.

In summary, these initial studies demonstrate the feasibility of directly observing the release and activity of renin from the JGA. This approach offers the opportunity to further our understanding of the mechanism, regulation, and dynamics of renin granule exocytosis from the JGA.

ACKNOWLEDGMENTS

We thank Dr. Kent T. Keyser, director of the UAB High Resolution Imaging Facility, for support and access to the multiphoton site. The support and advice of Dr. P. Darwin Bell are greatly appreciated.

GRANTS

This work was supported by grants from the National Institute of Diabetes and Digestive and Kidney Diseases (1HL650-01A1), the American Heart Association (SDG-0230074N), an American Society of Nephrology Carl W. Gottschalk Research Scholar Award, and Aventis Pharma Deutschland (to J. Peti-Peterdi).

REFERENCES