VESICLE-ASSOCIATED MEMBRANE PROTEIN-2 (VAMP2) MEDIATES cAMP-STIMULATED RENIN RELEASE IN MOUSE JUXTAGLOMERULAR CELLS

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Renin is essential for blood pressure control. Renin is stored in granules in juxtaglomerular (JG) cells, located in the pole of the renal afferent arterioles. The second messenger cAMP stimulates renin release. However, it is unclear whether fusion and exocytosis of renin-containing granules is involved. In addition, the role of the fusion proteins, SNAREs, in renin release from JG cells has not been studied. The vesicle SNARE proteins VAMP2 and VAMP3 mediate cAMP-stimulated exocytosis in other endocrine cells. Thus, we hypothesized that VAMP2 and/or 3 mediate cAMP-stimulated renin release from JG cells. By fluorescence-activated cell sorting, we isolated JG cells expressing green fluorescent protein, and compared the relative abundance of VAMP2/3 in JG cells versus total mouse kidney mRNA by quantitative PCR. We found that VAMP2 and VAMP3 mRNA are expressed and enriched in JG cells. Confocal imaging of primary cultures of JG cells showed that VAMP2 (but not VAMP3) co-localized with renin-containing granules. Cleavage of VAMP2 and VAMP3 with Tetanus toxin blocked cAMP-stimulated renin release from JG cells by ~50%, and impaired cAMP-stimulated exocytosis by ~50%, as monitored with FM1-43. Then, we specifically knocked-down VAMP2 or VAMP3 by adenoviral-mediated delivery of short hairpin silencing RNA. We found that silencing VAMP2 blocked cAMP-induced renin release by ~50%. In contrast, silencing VAMP3 had no effect on basal or cAMP-stimulated renin release. We conclude that VAMP2 and VAMP3 are expressed in JG cells, but only VAMP2 is targeted to renin-containing granules, and mediates the stimulatory effect of cAMP on renin exocytosis.

Renin has been a target of study for the past 100 years. It is the rate limiting enzyme in the generation of angiotensin II (ANGII) and is therefore essential for the regulation of blood pressure and also for renal development (1, 2). In JG cells, renin is processed from a pre-pro-renin precursor (3, 4, 5, 6) and stored in its active form in large dense core granules ranging from 0.8 to 1.2 µm size (7). Renin is released in response to low perfusion pressure or upon stimulation of JG cells with cAMP-elevating hormones (β-adrenergic receptors, prostaglandin E2) (7). Opposite to most secretory cells, renin release is stimulated by a decrease in intracellular calcium which augments intracellular cAMP in JG cells (“calcium paradox”) (8, 9). Despite the abundant literature in understanding the processing of renin from its precursor form (6, 10), sorting into granules (3, 11, 12), expression throughout development (13, 1, 14) and hormonal regulation of release and cross-talk signaling mechanism with other kidney cells (7, 15, 16, 17, 18), fundamental questions on how renin is released from JG cells and the proteins and molecular mechanisms involved have not been studied.

Earlier electron microscopy studies revealed that after stimulation of renin release, frequent contact areas of mature electron-dense renin containing granules with the plasma membrane could be observed in JG cells (19). In addition, membrane capacitance and imaging studies indicated that exocytosis is stimulated in JG cells by the cAMP and protein kinase-A (PKA) signaling pathway (20, 21). However, these results are indirect and direct evidence that renin release occurs via exocytosis is required.

In most secretory cells, exocytosis and membrane fusion are mediated by SNAREs [termed for SNAP (soluble N-ethylmaleimide-sensitive...
factor attachment proteins] receptors (22, 23, 24, 25). To mediate fusion, three SNARE families form a complex by direct interaction through their coiled-coil domains: one vesicle associated membrane protein (VAMP), one syntaxin, and one synaptosome associated protein (SNAP) (22, 26). In all cells studied SNARE complex zippering and exocytosis requires calcium. Each of these SNARE families is composed of several isoforms, which exhibit specific subcellular distribution (e.g. endosomal, Golgi, plasma membrane, etc). The SNARE hypothesis proposes that a tight selectivity for their pairing between VAMPs, syntaxins and SNAPs isoforms confer defined specificity to the intracellular trafficking events (27, 28, 29) and is specific to differential stimulatory triggers(30). Thus identification of the SNARE isoforms involved in the different steps of granule exocytosis after agonist stimulation is essential for understanding the potential targets that regulate cell type specific hormone release.

In the kidney, specific SNARE isoforms are expressed, i.e., VAMP2 and VAMP3 (31), syntaxin 3 and -4 (32, 33) and SNAP-23 (34, 35). In addition, in particular nephron segments, VAMP2 and VAMP3 have been implicated in cAMP-stimulated exocytosis (31, 36, 25, 37). Despite this evidence the involvement of SNAREs in renin release may be challenged by the inhibitory effect of intracellular calcium on JG cells which opposes the requirement of calcium for SNARE zippering and exocytosis. The expressions of VAMPs and other SNAREs in JG cells and their roles in renin release have not been previously explored.

In the present study we tested whether SNAREs are present in JG cells and the specific role of VAMP2 and VAMP3 in cAMP-stimulated renin release. We found that several members of the SNARE family are present in JG cells. Specific deletion of VAMP2 or VAMP3 proteins revealed a novel and specific role for VAMP2, but not VAMP3, in stimulated renin release and exocytosis. Therefore, stimulated renin release occurs via exocytosis requiring the SNAREs fusogenic machinery with a preferential selectivity for the vesicle protein VAMP2. By implicating VAMP2 in cAMP stimulated renin release and exocytosis our study provides evidence that renin release in JG cells occurs via exocytosis.

**Experimental Procedures**

**Isolation and primary culture of mouse JG cells.** Primary cultures of mouse JG cells were prepared following a protocol previously described and characterized with slight modifications (9, 38). In brief, 8-9 week-old C57/BL6 mice (Jackson Laboratories) were sacrificed by cervical dislocation. Kidneys were removed, decapsulated, and the renal cortex dissected. Combined cortical tissue from 4 mice was minced and then incubated with gentle stirring in a digestion buffer containing (in mM): 130 NaCl, 5 KCl, 2 CaCl₂, 10 glucose, 20 sucrose, and 10 HEPES (pH 7.4) along with 0.25% trypsin (Sigma Aldrich) and 0.1% collagenase type A (Roche Diagnostics) at 37°C for 45 min (9). The cell suspension was separated in 25ml of 40% isosmotic Percoll density gradient (Sigma) for 30 min centrifugation at 4°C and 27,000g using an SS-34 rotor/ Sorvall RC 5C Plus centrifuge. Cells were maintained at 37°C and 5% CO₂ in Dulbecco’s Modified Eagle Medium (DMEM, Invitrogen) supplemented with fetal calf serum and antibiotics (9). Culture dishes were coated with a freshly prepared poly-D-lysine solution (0.1mg/ml; Millipore). All protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of Henry Ford Hospital and RPCI in accordance with the National Institutes of Health Guidelines for Care and Use of Laboratory Animals. **Stimulation of renin release/cell treatment.** Before stimulation of renin release, JG cells were serum deprived for 2 hrs by replacing the medium with DMEM-serum free medium containing 100 U/ml penicillin and 100 µg/ml streptomycin. Renin release was stimulated by increasing intracellular cAMP with Forskolin (10 µM) plus IBMX (0.5 mM) for 1 hr. To measure renin release, the medium was collected, centrifuged to remove cellular debris and supernatants stored at -20°C until processed. To measure renin content in cells, 0.5 ml of 0.1% of Triton-X in phosphate buffered saline (PBS) was added to the plates; rocked for 10 minutes and then, cells were scraped, collected, spun at 16,000 g and supernatants stored at -20°C until processed. **Measurement of renin release and renin content.** Samples were incubated at 37°C for 3 hours with excess rat angiotensinogen in a buffer (pH 6.5) containing 0.1M phosphate buffer, 0.06M disodium EDTA, 0.2% gelatin, 0.1% neomycin sulfate and 0.1 mg/incubation of phenylmethyl sulfonyl fluoride (PMSF). Generated angiotensin I (ANGI) was measured using a Gamma Coat radioimmunnoassay kit (Diasorin) following manufacturer’s instructions. Values for renin concentration (ng ANGI* HR incubation) in cell culture medium (A) were
normalized to renin concentration in the cell lysate (B) and expressed as a percentage of renin content (A/B x 100). Validation experiments showed that <10% of the substrate is consumed under these conditions and the reaction maintains linearity of generated product over time.

**Western blot.** To detect VAMP2 and 3, cultured JG cells were lysed in a buffer containing (in mM): 150 NaCl, 50 HEPES (pH 7.5), 1 EDTA (pH 8), 2% Triton-X-100, 0.2% SDS, and a cocktail of protease inhibitors (39). Protein content was measured using a colorimetric assay (BSA, Pierce). Proteins were resolved in 12% SDS-polyacrylamide gels and transferred to a PVDF membrane (Amersham). Membranes were incubated first in blocking buffer containing 50 mM Tris, 500 mM NaCl, 0.1% Tween-20 (TBS-T) and 5% nonfat dried milk followed by primary antibody (mouse VAMP2 1/2000 or rabbit VAMP3 1/4000; Synaptic Systems)(40, 41, 42). Membranes were then washed in TBS-T and incubated with secondary antibody conjugated to horseradish peroxidase (anti-mouse or anti-rabbit 1/4000; Amersham). The signal was detected with a chemiluminescence kit (Amersham). As an internal loading control, membranes were re-blotted with an antibody to detect the housekeeping gene Actin (Santa Cruz).

**Cleavage of VAMP2/3 with Tetanus toxin.** Intact JG cells were pre-incubated for 19 hrs in DMEM-SF with either vehicle or 60 nM Tetanus toxin (Calbiochem). After the pre-incubation period, cells were lysed, SDS resolved in 12% polyacrylamide gels and VAMP2 and -3 detected by Western blot (mouse monoclonal antibody for VAMP2 [Synaptic System] and a rabbit polyclonal for VAMP3 [Abcam/Cambridge, UK]). VAMP2 (~116 aa) and VAMP3 (~103 aa) are selectively cleaved by Tetanus toxin at the C-terminal portion at position ~76 aa, releasing the 1-76 N-(amino)-terminal portion to the cytosol which is then degraded(43, 44, 45, 46). The antibody used to detect VAMP2 is directed against the N-terminus (2-17 aa), and therefore does not recognize the cleaved C-terminus portion of VAMP2. The antibody to detect VAMP3 is directed against aminoacids 1-100. Thus, effective Tetanus toxin cleavage is reflected as a decrease in VAMP2 and VAMP3 band intensity as previously reported (47, 48, 49). A second set of cells pre-treated with Tetanus toxin was subsequently stimulated with Forskolin plus IBMX for renin release studies.

**Immunofluorescence and confocal microscopy for VAMP2 and VAMP3 in mouse JG cells.** JG cells were grown on poly-D-lysine coated glass cover slips for 48 hrs. Four percent paraformaldehyde-fixed cells were permeabilized with 0.1% Triton-X in PBS for 10 min and then blocked in TBS-T/5% albumin. Cells were incubated with a FITC-labeled goat anti-renin antibody (Innovative Research) for 1 hr, followed by 1 hr incubation with an antibody for VAMP2 or -3 (Synaptic System; 1/100). After 3x washing, cells were incubated with a secondary antibody (anti rabbit IgG-Alexa Fluor 568; 1:200) washed with TBS-T, and mounted with Fluoromount-G (SouthernBiotech). Images were obtained using a laser scanning confocal imaging system (Visitech International) with excitation lasers 488nm (Renin) and 568nm (VAMP2 and -3). Fluorescence was filtered with 525lp and 590lp emission filters respectively. Cells were imaged with a 100X oil immersion lens (NA 1.33), 25 µm slit, at a pixel size of 0.05 µm (200X final magnification) in serial optical sections (0.3 µM) in the z-axis plane of the cells. Control experiments showed null cross-bleed between 488 and 568 channels under the conditions used for imaging. Tagged image file format (TIFF) images were deconvolved using the same settings (blind deconvolution, subpixel processing, 20 iterations) and co-localization analyzed using Autoquant software. Samples incubated in the absence of primary antibodies were used as control for non-specific binding. In addition, an extra sample was mounted without antibodies as control for autofluorescence.

**Isolation of JG cells from Ren-GFP transgenic mice by FACS.** To study the gene expression of VAMP2 and VAMP3, a pure population of JG cells was prepared from mice expressing green fluorescent protein (GFP) under the control of all regulatory elements of the Ren1c gene, a transgene described in detail previously (50). 3-5 adult female mice were euthanized and kidneys removed and stored in ice cold Hanks buffered salts solution (HBSS). Tissue was finely minced and suspended in 2 ml of HBSS, washed in HBSS and resuspended in 1 ml of enzyme mix: 2U/ml Blendzyme 3 (Roche), 0.25% collagenase XI (Sigma), 1.2U/ml elastase (Roche) and 100U/ml DNase I (Roche) in HBSS. Tubes were tumbled at 37°C for 30 minutes followed by Dounce homogenization (30 strokes). The suspensions were washed two times with ice cold HBSS and resuspended in ice cold solution (RPMI 1640 mixed with 2% fetal bovine serum and 100
U/ml of DNase I) until FACS sorting. Renin-expressing cells were sorted using a Becton-Dickinson FACS Aria cell sorter at 488 nm excitation and 530/30 bandpass emission filter. Autofluorescence was analyzed on transgene negative samples to establish limits of background fluorescence. The number of JG cells obtained was on the order of 50,000 per adult kidney pair.

**Quantitative PCR.** cDNA from FACS isolated JG cells and total kidney homogenate was prepared with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer’s protocol. Sybr-green based qPCR was carried out using the BIO-RAD iQ™SYBR®Green Supermix kit. Reactions were performed on a BIO-RAD CFX96 Real-Time PCR Detection System using the pre-programmed standard 2-cycle protocol with melting curve analysis. Primers were designed using Beacon Designer 3.01 (PREMIERE Biosoft International) to span introns and yield amplification fragments of 100-200bp (Table 2). Beta-2-microglobulin (B2M) was used as the reference gene. At the end of PCR cycling, melting curve analyses were performed and representative PCR products were run on agarose gels and visualized by ethidium bromide staining. Threshold cycle values (Ct) for the internal controls B2M were subtracted from amplified genes Ct values (delta Ct (ΔCt) value). To calculate the fold-enrichment factor in JG cells versus kidney homogenate, a relative quantitation method (ΔΔCt) (51) was used; were ΔΔCt represents [(ΔCt gene studied in JG cells)-(ΔCt of gene studied in total kidney homogenate)].

**Live- single cell monitoring of exocytosis using FM1-43.** FM1-43 fluoresces only when bound to membranes, but not in solution (52, 39). Therefore the newly inserted granule membrane that occurs after exocytosis can be monitored and correlated with the increased fluorescence in JG cells bathed with FM1-43. JG cells were grown on coverglass and mounted in a 37°C-temperature controlled chamber on an inverted confocal microscope. 4 μM FM1-43 was added to the flowing bath (cell culture media) for the duration of the experiment. FM1-43 was excited at 488 nm, and emission was collected with a 525-nm long-pass emission filter. Images were acquired with a laser scanning confocal microscopy for 30 ms, every 10s. Typically 3 to 5 minutes elapsed until fluorescence intensity at the plasma membrane reached a plateau. After a 2 min baseline period with continuous FM1-43 perfusion, Forskolin plus IBMX were added to the bath and images recorded for a 10 min period after which FM1-43 was washed out with fresh media containing the quencher bromophenol blue (1 mM) (53). The experimental recordings obtained were analyzed with Metamorph software by designating a region of interest (ROI) encompassing the plasma membrane, yielding average fluorescence intensity throughout the course of the experiment. Fluorescence intensity in each ROI was normalized to average baseline value set at 100 (n = total number of cells studied).

**Construction of adenoviral vectors and transduction of primary cultures of mouse JG cells.** For adenoviral particle production encoding silencing RNA VAMP2 (VAMP2 shRNA), oligonucleotide fragment encoding 19 nucleotides (nt) of sense VAMP2 (5′-GCTCAAGCGCAAATACTGG-3′) followed by a loop region (TTCAGAGA) and the antisense of the 19 nt was subcloned into the 5′ AflIII and 3′ SpeI sites of the Adenovector-pMIGHTY (Viraquest, North Liberty, IA). Oligonucleotides were generated to encode AflII and SpeI sites at the 5′ and 3′ ends, respectively, for convenient insertion into the Adenovector-pMIGHTY. The control construct (Scrambled shRNA) was generated similarly, using a scrambled 19 nt sequence (5′-TTCTCCGAACGTGTCACGT-3′). Adenoviral particle production encoding silencing RNA VAMP3 (VAMP3 shRNA), was constructed encoding the sequence: 5′-GGATCTTCTTCTCGAGACTTTT-3′. All constructs were sequenced before viral particles were produced.

For adenoviral-mediated knockdown of VAMP2 and VAMP3, 24 hrs after plating, primary cultures of mouse JG cells were incubated in DMEM-serum free containing VAMP2 shRNA or VAMP3 shRNA particles (100PFU/cell). After 3 hrs, fetal calf serum was added to the cultured media to reach a 5% concentration and incubated at 37°C for another 28 hrs. JG cells were then stimulated with F/IBMX for 1 hr as described above.

**Reagents.** Fetal calf serum was obtained from Hyclone, DMEM culture medium and antibiotics from Invitrogen. Forskolin, IBMX, Percoll and protease inhibitors from Sigma Aldrich (St Louis, MO). Tetanus toxin was from Calbiochem and FITC-labeled binding domain Tetanus toxin was from List Biological Laboratories (San Jose, CA). We obtained poly-D-lysine from Millipore. Radioimmunoassay kits to measure ANGI from
Diasorin (Stillwater, MN). FM1-43 and Alexa-Fluor conjugated antibodies were from Invitrogen.

Statistical Analysis. Data were expressed as mean ± SE and subjected to one-way ANOVA with multiple comparisons made by the Student-Newman-Keuls Method. A value of \( p < 0.05 \) was considered significant.

RESULTS

Expression of SNARE proteins in FACS isolated JG cells.

Granule exocytosis and membrane fusion are mediated by SNAREs. However, the expression of the minimal SNARE exocytic machinery in the renal vasculature and in JG cells has not been studied. The vesicle SNAREs VAMP2 and VAMP3 have been previously reported to mediate cAMP-stimulated exocytosis in renal epithelial cells (31, 36, 37, 25). Thus we first studied their expression in a pure population of JG cells isolated from adult transgenic mice expressing green fluorescent protein (GFP) by FACS (50) and compared it to their relative abundance in total kidney homogenates. Our quantitative PCR results showed that VAMP2 and VAMP3 genes are expressed in freshly isolated JG cells and total kidney (Figure 1). The relative mRNA abundance (fold-enrichment factor) from JG cells compared to total kidney mRNA from quantitative PCR was: VAMP2 (6.2±1-fold), VAMP3 (3.4±0.5-fold) (Table 1). Since renin is only expressed in JG cells under normal conditions, renin mRNA abundance was used as a control of JG cell purity. As expected, in JG cells isolated from adult mice, renin mRNA was enriched 3346±345-fold when compared to total kidney homogenates. These data indicate that JG cells express VAMP2 and VAMP3 mRNAs, and their mRNA levels are relatively enriched in JG cells when compared to the rest of the kidney.

The expression of the minimal SNARE fusion machinery in JG cells has not been studied. As shown in Table 1, additional VAMPs isoforms (VAMP4, -5, -7 and -8), as well as several isoforms of its cognate binding partners Syntaxin (Syntaxin1, -2, -3 and -4) and SNAP23 were found to be expressed in FACS-isolated JG cells. SNAP25 was not detected in JG cells or total kidney homogenates.

VAMP2 and VAMP3 proteins are expressed in JG cells and are differentially localized in JG cells.

To start addressing the possible role of VAMP2 and/or VAMP3, we studied VAMP2 and VAMP3 protein expression and localization within JG cells. By Western blot analysis of primary cultures of JG cells we observed a band corresponding to the expected molecular weights of 18 kDa (VAMP2) and 11 kDa (VAMP3) (Figure 2A, lane 2 in both panels). Brain homogenate was used as a positive control (Figure 2A, lane 1 in both panels). To directly monitor the expression and subcellular localization of VAMP2 and VAMP3 in JG cells, we performed dual labeling immunofluorescence and confocal microscopy for renin (54) and VAMPs. As shown in Figure 2B (top panel) we found that VAMP2 is expressed in JG cells in large granules that occupy most of the cytoplasm that are also stained for renin, indicating a high degree of co-localization. Different from VAMP2, VAMP3 was found in smaller vesicles and in a peri-nuclear area. We observed little to no co-localization of VAMP3 with renin-containing granules (Figure 2B, bottom panel). Therefore, both VAMP2 and VAMP3 are expressed in JG cells but only VAMP2 co-localized with renin-containing granules.

Cleavage of VAMP2 and 3 by Tetanus toxin inhibits cAMP-stimulated renin release in JG cells.

To test whether VAMP2 and/or VAMP3 are involved in cAMP-stimulated renin release we first used Tetanus toxin. Previous studies showed that cell permeabilization and treatment with the proteolytic chain of tetanus toxin (light chain) specifically cleaves and inactivates VAMP2 and -3 (44, 46, 56, 55). Maximal stimulation of JG cells results in the release of only ~4% of the total renin content. Therefore permeabilization induces “leakage” of renin and is not feasible in JG cells. Thus we first tested whether Tetanus toxin is efficiently internalized by intact JG cells (non-permeabilized). Incubation with a fluorescent labeled membrane binding portion of Tetanus toxin (FITC-labeled C-fragment) was sufficient to efficiently label most JG cells, indicating internalization of the toxin (Figure 3A). We then tested whether the intact Tetanus toxin efficiently cleaves VAMP2 and -3 in intact JG cells. After 19 hrs incubation with Tetanus toxin (60 nM) or vehicle, VAMP2 and VAMP3 protein levels were analyzed by Western blot. As shown in Figure 3B, VAMP2 (left panel) and VAMP3 (right panel) protein was significantly decreased 40±10% and 35±10% respectively (CONT=1; VAMP2= 0.6±0.1; VAMP3= 0.65±0.1; n = 8; \( p < 0.05 \)). Finally, we studied whether inactivation of VAMP2/3 in JG cells with Tetanus toxin blocks cAMP-stimulated renin release. After pre-treatment of JG cells with...
Renin release from renal JG cells is essential for kidney development and the control of blood pressure. While the hormonal and paracrine control of renin release has been extensively studied, the proteins involved and the biological mechanism mediating renin release have not been identified. Moreover, whether renin release from granules requires fusion with the plasma membrane has not been directly demonstrated. In several endocrine and
secretory cells, specific SNARE proteins mediate granule exocytosis and peptide/hormone release (57, 58, 59). However, the expression and role SNAREs in renin release have not been addressed. One of the reasons why SNAREs and fusion were not explored might be the paradoxical inhibitory effect of calcium on renin release that opposes the necessary role of calcium on SNARE complex zippering. Our data show for the first time that the SNARE machinery is expressed in JG cells and the VAMP isoform VAMP2 localizes to renin-containing granules and mediates cAMP-stimulated renin release. These are the first data identifying a trafficking protein involved in renin release. More importantly, the requirement of the SNARE protein, VAMP2, on renin release implies that the renin granule undergoes exocytosis.

We observed differential localization of VAMP2 and VAMP3 in JG cells. Most VAMP2 localized to renin-containing granules whereas VAMP3 was excluded from renin granules and present in smaller vesicles and in a peri-nuclear pattern. While our data indicate that VAMP3 is not involved in constitutive or regulated renin release, VAMP3 may play additional roles in JG cells. Similar to our results, in other exocrine cells, VAMP3 was found to be mainly localized to organelles involved in the biosynthetic trafficking pathway rather than in controlling final steps of vesicle or exocytosis (27).

To study the role of VAMP2 and -3 on renin release we first used the clostridial Tetanus toxin. Tetanus toxin recognizes and cleaves a sequence uniquely present in VAMP2 and -3 (55, 46, 44, 56). In intact (non-permeabilized) JG cells, Tetanus toxin was efficiently internalized and cleaved approximately 50% of the total pool of VAMP2 and VAMP3. This effect was sufficient to block cAMP-stimulated renin release by 50 to 60%. It is possible that incomplete VAMP2 or VAMP3 inactivation explains the partial effect of Tetanus toxin. However, higher concentrations (100 nM) of the toxin did not further inhibit renin release (data not shown). Tetanus toxin inhibition of renin release did not affect processing of renin from its pro-form since total renin content in JG cell lysates did not change when compared to controls. The role of VAMP2 on exocytosis rather than renin processing is further supported by the inhibition of cAMP-stimulated exocytosis, as measured with FM1-43 membrane labeling.

To specifically address the role of these VAMPs we knocked down VAMP2 and VAMP3 by shRNA. Gene silencing in primary cells is difficult, thus we used adenoviral vectors with VAMP2 or VAMP3 shRNA sequences. Our data shows that a 50-60% protein knockdown of VAMP2 inhibits cAMP-stimulated renin release by 50%, whereas silencing VAMP3 with similar efficacy had no effect. While these data clearly support a role of VAMP2 in mediating renin release we cannot rule out the involvement of other VAMPs. It is possible that VAMP7 or 8 (which are not cleaved by Tetanus toxin) may be involved in mediating part (30-50 %) of the stimulatory effect of cAMP on granule fusion with the plasma membrane. This would imply that there may be at least 2 pools of granules containing mature renin, perhaps with different SNARES. However it is also possible that upon decrease or inactivation of VAMP2 other VAMP isoforms exert a compensatory role as it occurs in other secretory cells (60). Taken together our data demonstrates that VAMP2 but not VAMP3 mediates most of the cAMP-stimulated renin release although the partial involvement of other isoforms cannot be ruled out and will require further investigation.

Baseline (non-stimulated) renin release was not affected by either Tetanus toxin or by selective knockdown of either VAMP2 or VAMP3. These data indicate that VAMP2 participates in the regulated rather than the constitutive release of renin or in its maturation process. It is likely that different VAMPs mediate the constitutive and the regulated renin pathways as reported in other exocrine cells (29, 22). In addition, we found that inactivation or knockdown of VAMP2 and VAMP3 with Tetanus toxin or silencing RNA did not affect the amount of total renin in JG cells (renin content). Thus, the inhibitory effect of silencing VAMP2 is not likely to due to a decrease in the amount of mature renin in JG cells, or defective granule maturation. Rather our data points to VAMP2 being necessary for granule-plasma membrane fusion (exocytosis per se).

In vivo, the amount of renin released into the circulation represents a very small percentage of the total renin content (61). Renin release from JG cells in isolated afferent arteriole is approximately 2 to 5 % of the total renin content (15). For our experiments, we have used an extensively characterized preparation of primary JG cells that is at least 80% pure and responds to physiological stimuli (9). While the percent of total renin released may seem small, our data are in excellent agreement.
with previous work studying JG cells *in vivo* or after isolation (15, 62, 63, 64). This magnitude of response is similar to most endocrine cells, which release a small fraction of it granular contents upon stimulation (24, 65). We found that culturing JG cells for up to 72 hours did not result in dedifferentiation, since renin release values under constitutive or stimulated conditions, were comparable to those of cells cultured for 24 or 48 hrs. In addition, longer incubation times and viral transduction *per se* did not affect renin release nor renin content values.

At least two other SNARE proteins, a syntaxin and SNAP, are required to mediate granule exocytosis. The expression of these SNAREs has not been studied in JG cells. In other endocrine cells where exocytosis is stimulated by cAMP, VAMP2 pairs primarily with syntaxin -1, -3, -4 and SNAP23. We found that syntaxin-1, -2, -3 and -4 and SNAP23 (but not SNAP25) are present in JG cells. In addition we also found that other VAMP isoforms (VAMP4, -5, -7 and -8) as well as the accessory proteins Munc18 (Munc18a, -b and -c) are expressed in JG cells (Table 1). While the full fusogenic machinery seems to be present in JG cells, we did not find a differential pattern of enrichment for those SNAREs in JG cells. This is somehow predictable since it is likely that the different SNAREs are involved in different biological processes within the JG cells as well as kidney cell types. Furthermore, some of the isoforms that we found to be expressed in JG cells may be involved in renin granule maturation. Thus it is essential to study the VAMP2-interacting SNARE proteins individually and their specific involvement on cAMP-stimulated renin granule exocytosis.

The molecular mechanisms by which cAMP increases renin release *via* VAMP2 are not known. Previous studies have shown that SNARE complex and exocytosis can be regulated directly by phosphorylation in other secretory cells (66, 67, 57). It is possible that cAMP *via* PKA could be directly responsible for phosphorylation of VAMP2 binding partners such as SNAP23 (67, 57). In addition there are several proteins that modulate SNARE-mediated exocytosis such as snapin, tomosyn and complexins (68, 69, 70) that are targets for the cAMP-PKA pathways. Any of these may be present in JG cells and could subsequently enhance fusion of pre-docked dense core renin granules to the plasma membrane, but these have not been studied.

In most cells studied, SNARE zippering and complex formation, which mediates the ultimately step in membrane fusion, requires calcium (22). In addition, in most secretory cells studied, calcium is the triggering second messenger for exocytosis (22, 71). Opposite to most cells, in JG cells, a decrease in intracellular calcium stimulates renin release, whereas high calcium inhibits it, a phenomenon known as the calcium paradox (7). While it might seem contradictory that SNAREs mediate renin release with an opposing effect of intracellular calcium on secretion there are several potential explanations. One possible explanation is that a localized calcium spike or calcium microdomain occurs at the plasma membrane specifically near fusion sites without affecting intracellular calcium levels or stores (72). In addition some SNAREs are less sensitive to the requirement for calcium to induce exocytosis. For example SNAP23 is less sensitive to changes in calcium concentration than SNAP25 (73). Synaptotagmins, which are SNARE complex modulatory proteins, serve as calcium sensors (74). Another possible explanation could be that the synaptotagmin isoforms mediating renin release in JG cells are calcium independent as described earlier (74). Thus it is possible that the VAMP2 mediated renin release involves primarily a set of SNAREs that do not require an increase in calcium for zippering. However, this remains speculative and requires future investigation.

From all of the hypertensive patient population, less than thirty percent possess the expectedly low plasma renin activity (75, 76). The remaining percentage exhibits either normal or high plasma renin values both of which are unexpected or “abnormal” since renin should be suppressed by higher blood pressure. The mechanisms that mediate this higher than normal renin levels in hypertension and whether they involve enhanced renin release and alterations in the proteins mediating renin secretion or signaling in JG cells have not been elucidated. Our findings identify for the first time that the SNARE protein VAMP2 but not VAMP3 mediate cAMP-stimulated renin release. Future studies focusing on the selective SNARE pairs from the syntaxin family and the mechanisms that modulate SNARE complex formation in JG cells would advance our understanding of renin exocytosis and may provide novel targets for inhibition of renin release.
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FOOTNOTES
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The abbreviations used are: SNARE, SNAP (soluble N-ethylmaleimide-sensitive factor attachment proteins) receptors; VAMP2, vesicle associated membrane protein-2; VAMP3, vesicle associated membrane protein-3; B2M, Beta-2-microglobulin; DMEM, Dulbecco’s Modified Eagle Medium.

FIGURE LEGENDS
Table 1. Real-time PCR of SNAREs in JG cells. ΔΔCt was calculated as:
\[\Delta \Delta Ct = (\Delta Ct \text{ in JG cells}) - (\Delta Ct \text{ in total kidney homogenates})\];
where (ΔCt) is Threshold value cycle (Ct) of gene studied minus Ct from the internal control B2M. Fold-enrichment factor of genes in JG cells versus total kidney was calculated as 2-ΔΔCt as previously reported (51). SNAP25 was not included in the table since not amplification was detected. Values are mean ± SE; n = 3. Negative ΔΔCt values (Syntaxin3 and Munc18b) represent a fold enrichment <1 indicating a higher abundance of these genes in total kidney versus JG cells.

Table 2. qPCR Primer Sequence and amplicon sizes for SNAREs. Primers were designed using Beacon Designer 3.01 (PREMIERE Biosoft International) to span introns and yield amplification fragments of 100-200 bp.

Fig. 1. Representative image of quantitative PCR products for VAMP2 and VAMP3. PCR products were resolved in an agarose gel stained with ethidium bromide (n = 3). Lane 1 is molecular weight ladder. Lanes 2-5 are PCR products from total kidney homogenate illustrating B2M, renin, VAMP2 and VAMP3 respectively. Lanes 6-9 correspond to PCR products from freshly isolated JG cells B2M, renin, VAMP2 and VAMP3 respectively. Some lanes not relevant to the current study have been intentionally cut out of the picture.

Fig. 2. Expression and subcellular localization of VAMP2 and VAMP3 in JG cells. A. Representative Western blot showing expression of VAMP2 (18 kDa) and VAMP3 (11 kDa) in a JG cell lysate. Top panel is VAMP2, bottom panel is VAMP3. Lane 1 is brain homogenate (2.5 µg) used as a positive control, lane 2...
is JG cell lysate (7.5 µg) \((n = 4)\). B. Immunofluorescence and confocal microscopy of VAMP2 and renin on a single mouse JG cell. Top left panel shows a representative image from 5 preparations of a single JG cell labeled with an antibody against renin (green), 32 confocal slices (z-step 0.3 µm) were stacked into one image projection. Large renin-containing granules that range in size 0.8-1.5 µm can be observed. Middle panel shows VAMP2 labeling (red) in the same cell. Right panel shows a merged image illustrating co-localization of renin with VAMP2 as illustrated by yellow-orange color \((n = 5\) different preparations). Bar 3 µm. C. Immunofluorescence and confocal microscopy of VAMP3 and renin on a single mouse JG cell. Bottom left panel shows an individual JG cell immunolabeled with renin (green); middle bottom panel (red) is the same JG cell labeled with VAMP3 antibody; bottom right panel is the merged image. No co-localization is observed between small VAMP3 labeled vesicles and renin granules.

Fig. 3. Internalization and cleavage of VAMP2 and VAMP3 by Tetanus toxin. A. Tetanus toxin is efficiently internalized in intact JG cells. JG cells were incubated for 19 hrs with either vehicle (CONTROL; left panel) or FITC-labeled receptor binding domain of Tetanus toxin (C-fragment; List Biological Laboratories) (right panel). After fixation and mounting, incorporated fluorescence was analyzed by confocal fluorescence imaging. B. Representative Western blots showing efficient cleavage of VAMP2 and VAMP3 by Tetanus toxin. Left panel is VAMP2 and right panel is VAMP3 protein expression. JG cells were incubated with either vehicle (lane 1 in both panels) or Tetanus toxin (lane 2 in both panels). After treatment, JG cells were lysed, SDS-PAGE resolved in 12% polyacrylamide gel and transferred to PVDF membranes, which were subsequently blotted with VAMP2 or VAMP3 antibodies respectively. Note that a decrease in VAMP2 and VAMP3 band intensity reflects efficient cleavage by Tetanus toxin. Membranes were re-blotted with an antibody against GAPDH as an internal loading control (M.W= ~35 kDa). GAPDH signal was not different between groups \((p = N.S.)\).

Fig. 4. Effect of Tetanus toxin on cAMP-stimulated renin release and total renin content. A. Renin release. After pre-treatment of JG cells with vehicle (Black bars) or Tetanus toxin (Grey bars) as described in Figure 3, JG cells were serum deprived for 2 hrs followed by treatment with vehicle (Control) or forskolin (10 µM) plus IBMX (0.5 mM) (F/IBMX) for 1 hr to stimulate cAMP levels as indicated in X-axes. Cell culture supernatants were collected for measurement of renin release as described in Experimental Procedures. Attached cells in the culture dishes were lysed in 0.1% Triton X, centrifuged and supernatants collected for measurement of total renin content. Renin release is expressed as a percentage of the total renin content. Data is expressed as mean ± SE. \(*p < 0.01, \text{CONT vs Forskolin/IBMX}; \#p < 0.05, \text{Forskolin/IBMX vs Tetanus toxin+Forskolin/IBMX}; n = 4\). B. Effect of Tetanus toxin on total renin content in JG cells. Total renin content values are corrected by protein concentration (ng ANGI* HR incubation/mg protein). Data is expressed as mean ± SE \((n = 4; p = N.S.)\).

Fig. 5. Measurement of single-cell exocytosis using FM1–43. A. Representative pictures of two JG cell stained with 4 µM FM1–43. Top panels show fluorescence intensity after the addition of vehicle (2 minutes, Basal) or Forskolin plus IBMX (12 minutes, Stimulated). Bottom panels show fluorescence intensity in a JG cell pre-treated with Tetanus toxin under Basal (left) or cAMP-stimulated (right) conditions. Pictures were pseudocolored with rainbow scale (blue: low and red: high fluorescence intensity). B. Cumulative data for FM1–43 fluorescence intensity over time. Fluorescence intensity was normalized to initial intensity \((F_0)\) in JG cells treated with vehicle (Basal). Arrow indicates the addition of Forskolin plus IBMX. Fluorescence intensity was measured in regions of interest from \(n = 7\) cells from 4 independent JG cell preparations. Data is expressed as mean ± SE; \(*p < 0.05\) vs control group.

Fig. 6. Effective adenoviral delivery and specific knockdown of VAMP2 and VAMP3 in JG cells. A. Transduction efficiency in JG cells. JG cells were transduced for 24 hrs with an adenovirus encoding green fluorescent protein (GFP) under the control of cytomegalovirus promoter (Ad-CMV-GFP). No fluorescence was detected in CONTROL (non-transduced) cells. B. Comparison of cAMP-stimulated response in either
non-transduced (black bar) or JG cells transduced with Ad-CMV-GFP (grey bar). After transduction of JG cells for up to 48 hrs, cells were serum starved for 2 hrs and treated for 1 hr with Forskolin plus IBMX (10 µM/0.5 mM) or vehicle according to description in Experimental Procedures. Data shows stimulated renin release (Forskolin/IBMX stimulated renin release minus basal [vehicle-treated] renin release) expressed as percentage of renin content. Basal renin release values for non-transduced and transduced JG cells were 1.0±0.1 and 0.9±0.3 % of renin content respectively (n = 4; p = N.S.) Data is expressed as mean ± SEM. C. Representative Western blot illustrating effective silencing of VAMP2 (left panel). JG cells were transduced with adenoviral particles encoding shRNA for either a scramble sequence or VAMP2. 28 hrs after transduction, JG cells were lysed, SDS-PAGE resolved in 12% polyacrylamide gel and transferred to PVDF membranes, which were subsequently blotted with VAMP2 antibody. Membranes were re-blotted with an antibody against GAPDH as an internal loading control (M.W= ~35 kDa). Right panel shows quantification of VAMP2 protein expression expressed as a ratio of GAPDH signal (n = 4). Scrambled shRNA was arbitrarily set to 1. D. Representative Western blot illustrating effective silencing of VAMP3 (left panel, line 3) versus scrambled shRNA (line 1). Line 2 shows JG cells transduced with shRNA for VAMP2, which does not decrease VAMP3 expression. Right panel is quantification of VAMP3 protein expressed as a ratio of GAPDH signal (n = 3; p < 0.01).

Fig. 7. Effect of adenoviral delivery of short hairpin silencing for VAMP2 and VAMP3 on cAMP-stimulated renin release and total renin content in JG cells. A. Forskolin plus IBMX stimulated renin release is shown in JG cells transduced with scrambled (black bar), VAMP2 shRNA (grey bar) and VAMP3 shRNA (stripped bar). After transduction of JG cells for 28 hrs, cells were serum starved for 2 hrs and treated for 1 hr with Forskolin plus IBMX (10 µM/0.5 mM) or vehicle according to description in Experimental Procedures. Data shows stimulated renin release (Forskolin/IBMX stimulated renin release minus vehicle-treated (basal) renin release) expressed as percentage of renin content. Basal renin release values were no significant different: scrambled shRNA = 0.6±0.1; VAMP2 shRNA = 0.8±0.2; VAMP3 shRNA = 0.7±0.3; (p = N.S.). Data is expressed as mean ± SEM. B. VAMP2 and VAMP3 knockdown does not affect renin content. Total renin content values are corrected by protein concentration (ng ANGI* HR incubation/mg protein). Renin content from scrambled shRNA was arbitrarily set to 100. Data is expressed as mean ± SE (n = 4; p = N.S.).
Table 1.

<table>
<thead>
<tr>
<th>Gene</th>
<th>ΔΔCt</th>
<th>Fold-Enrichment factor</th>
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<tbody>
<tr>
<td>Renin</td>
<td>11.7</td>
<td>3346±345</td>
</tr>
<tr>
<td>VAMP2</td>
<td>2.6</td>
<td>6.2±1.0</td>
</tr>
<tr>
<td>VAMP3</td>
<td>1.7</td>
<td>3.4±0.5</td>
</tr>
<tr>
<td>VAMP4</td>
<td>1.6</td>
<td>3.1±0.3</td>
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<tr>
<td>VAMP5</td>
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<td>3.2±0.1</td>
</tr>
<tr>
<td>VAMP7</td>
<td>0.5</td>
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<tr>
<td>VAMP8</td>
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<tr>
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<td>Munc18b</td>
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<td>150</td>
</tr>
</tbody>
</table>
Figure 1

Total Kidney  JG cells

M.W. Ladder  B2M  Renin  VAMP2  VAMP3  B2M  Renin  VAMP2  VAMP3

100 bp  200 bp
Figure 2

A.

<table>
<thead>
<tr>
<th>Brain (+)</th>
<th>JG lysate</th>
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<td>(Lane 1)</td>
<td>(Lane 2)</td>
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VAMP2 (18 kDa)  
VAMP3 (11 kDa)
Figure 2

B.

Renin VAMP2 Merged

[Images of Renin, VAMP2, and Merged]

3 μm

C.

Renin VAMP3 Merged

[Images of Renin, VAMP3, and Merged]

3 μm
Figure 3

A.

Control

Tetanus toxin-FITC

B.

VAMP2

VAMP3

VAMP2 (18kDa)

VAMP3 (11kDa)

GAPDH (35kDa)

GAPDH (35kDa)

CONTROL  TT  CONTROL  TT
Figure 4

A.

![Graph showing renin release](image1)

B.

![Graph showing renin content](image2)
Figure 5

A.

B.

Vehicle  Forskolin/IBMX

Control  Tetanus toxin

Mean Fluorescence Intensity (% of F0)

Forskolin/IBMX

Control  Tetanus toxin

Time (minutes)

0 1 2 3 4 5 6 7 8 9 10 11 12

*
Figure 6

A. Ad-CMV-GFP

B. cAMP-stimulated renin release (% of renin content)

Non-transduced  Ad-transduced
Figure 6

C. VAMP2

VAMP2 protein/GAPDH (fold vs Scrambled shRNA)

VAMP2
(18 kDa)

GAPDH
(35 kDa)

Scrambled shRNA
VAMP2 shRNA

D. VAMP3

VAMP3 protein/GAPDH (fold vs Scrambled shRNA)

VAMP3
(11 kDa)

GAPDH
(35 kDa)

Scrambled VAMP2 VAMP3 shRNA shRNA shRNA

Scrambled shRNA VAMP3 shRNA

*
Figure 7

A.

![Bar graph showing cAMP-stimulated renin release (% of renin content) for Scrambled shRNA, VAMP2 shRNA, and VAMP3 shRNA with forskolin/IBMX treatment.](image)

B.

![Bar graph showing Renin content (% of Scrambled shRNA) for Scrambled shRNA, VAMP2 shRNA, and VAMP3 shRNA with forskolin/IBMX treatment.](image)
Vesicle-associated membrane protein-2 (VAMP2) mediates cAMP-stimulated renin release from mouse juxtaglomerular cells
Mariela Mendez, Kenneth W. Gross, Sean T. Glenn, Jeffrey L. Garvin and Oscar A. Carretero

J. Biol. Chem. published online June 27, 2011

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