Role of the SNARE protein SNAP23 on cAMP-stimulated renin release in mouse juxtaglomerular cells

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IN MAMMALS, RENIN IS STORED in electron-dense core granules in the juxtaglomerular (JG) cells. Therefore, the controlled mechanism involved in renin release is essential for the regulation of blood pressure. Exocytosis of renin-containing granules is likely involved in renin release; a process stimulated by cAMP. We found that the “soluble NSF (N-ethylmaleimide-sensitive factor) attachment protein receptor” (SNARE) protein VAMP2 mediates cAMP-stimulated renin release and exocytosis in JG cells. To mediate exocytosis, VAMP2 must interact with a synaptosome-associated protein (SNAP). In the renal cortex, the isoform SNAP23 is abundantly expressed. We hypothesized that SNAP23 mediates cAMP-stimulated renin release from primary cultures of mouse JG cells. We found that SNAP23 protein is expressed and colocalized with renin-containing granules in primary cultures of mouse JG cells. Thus, we then tested the involvement of SNAP23 in cAMP-stimulated renin release by transducing JG cells with a dominant-negative SNAP23 construct. In control JG cells transduced with a scrambled sequence, increasing cAMP stimulated renin release from 1.3 ± 0.3 to 5.3 ± 1.2% of renin content. In cells transduced with dominant-negative SNAP23, cAMP increased renin from 1.0 ± 0.1 to 3.0 ± 0.6% of renin content, a 50% blockade. Botulinum toxin E, which cleaves and inactivates SNAP23, reduced cAMP-stimulated renin release by 42 ± 17%. Finally, adenovirus-mediated silencing of SNAP23 significantly blocked cAMP-stimulated renin release by 50 ± 13%. We concluded that the SNARE protein SNAP23 mediates cAMP-stimulated renin release. These data show that renin release is a SNARE-dependent process.

molecular mechanisms and proteins involved in exocytosis of renin-containing granules.

In secretory cells, the process of exocytosis and membrane fusion is mediated by “soluble NSF (N-ethylmaleimide-sensitive factor) attachment protein receptors” (SNAREs) proteins (2), whose assembly and disassembly are further modulated by additional regulatory proteins (31). Once secretory cells are stimulated, a minimum of three SNARE family members interact to form a stable four-helix complex: one vesicle-associated membrane protein (VAMP), one syntaxin, and one synaptosome-associated protein (SNAP) (36). In neurons (30) and neuroendocrine cells, such as pancreatic beta cells (7, 15, 17, 37), VAMP-2, Syntaxin 1A, and SNAP25 are the putative SNARE proteins.

In nonneuronal secretory cells, VAMP2 remains the dominant secretory granule SNARE protein to mediate exocytosis. However, SNAP25 is replaced by several other SNAP isoforms in mediating exocytosis, which are ubiquitously expressed (3, 12, 13, 29). The isoform SNAP23 mediates the final step in regulated granule fusion with the plasma membrane in many nonneuronal secretory cells such as pancreatic acinar cells (6, 16), mast cells (9), and fat cells (21, 27). The renal cortex shows high levels of SNAP23 expression (20). Specifically, SNAP23 mediates cAMP-stimulated exocytosis in principal cells (20) and collecting ducts of the kidney (8). However, it is not known whether SNAP23 is present and mediates cAMP-stimulated renin release in JG cells.

We embarked on identifying the components of the exocytic machinery mediating renin-dense core granule exocytosis in JG cells. Recently, we identified VAMP2 as the VAMP isoform that mediates cAMP-stimulated renin release in JG cells (23). In the present study, we hypothesized and demonstrated that SNAP23 is present and involved in regulated release of renin in primary cultures of JG cells.

MATERIALS AND METHODS

Isolation and culture of mouse primary JG cells. Primary cultures of mouse JG cells were prepared as described and characterized before (23, 25). C57BL6 mice (8 to 9 wk old, Jackson Laboratories) were killed by cervical dislocation. Kidneys were removed, decapsulated, and the renal cortex was dissected. Cortical tissue from four mice was minced and digested as we described previously (23). Cells were cultured in DMEM containing 100 U/ml penicillin, 100 μg/ml streptomycin, and 5% fetal calf serum (HyClone) at 37°C/5% CO₂ in poly-D-lysine-coated plates (0.1 mg/ml; Millipore). All protocols were approved by the Institutional Animal Care and Use Committee of Henry Ford Hospital and in accord with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.
SNAP23 MEDIATES cAMP-STIMULATED RENIN RELEASE

F499

**Western blot.** To study the expression of SNAP23, 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 protease inhibitor cocktail (1). Protein content was measured by colorimetric assay (BIAcore, Pierce). Proteins were resolved on 12% SDS-PAGE 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 for 60 min and then with a primary antibody (1/15,000 rabbit SNAP23; Synaptic Systems) in blocking buffer for 60 min. Membranes were washed in TBS-T and incubated with a secondary antibody conjugated to horse-radish peroxidase (1/4,000 anti-rabbit; Amersham). For SNAP23, a monoclonal antibody was used at 1/5,000 dilution (Covance). VAMP2 (monoclonal), VAMP3, and VAMP4 antibodies (polyclonal) were used at a 1/3,000 dilution (Synaptic Systems). As an internal loading control, membranes were reblotted with an antibody against the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (1/100,000 GAPDH; Millipore). Signals were detected with a chemiluminescence kit (Amersham).

**Immunofluorescence and confocal microscopy.** JG cells were grown on poly-L-lysine-coated coverslips. Following fixation with 4% paraformaldehyde in PBS (pH 7.4) for 30 min, membranes were permeabilized with 0.1% Triton X-100 in PBS for 10 min and blocked in TBS-T/0.15% albumin for 30 min. Cells were incubated first with a FITC-labeled antibody against renin (1/250, Innovative Research) for 1 h. Then, cells were labeled for 1 h with the primary rabbit antibody for SNAP23 (1/100, Synaptic System), followed by 1-h incubation with an anti-rabbit secondary antibody (1/200 Alexa Fluor 568) and mounted with Fluoromount-G (SouthernBiotech). Images were obtained using a laser-scanning confocal imaging system (Visitech International) with 488-nm (renin) and 568-nm (SNAP23) excitation lasers. Fluorescence was filtered with 525- and 590-nm emission filters, respectively, and images were acquired with a cooled charge-coupled device camera (Hamamatsu). Fluorescence signals were measured using confocal microscopy software (Media Cybernetics). Images were obtained using two-dimensional blind deconvolution. Images from both channels were aligned; pixel-by-pixel colocalization measured using a minimum Mander’s overlap coefficient of 0.95 and an image for overlapping pixels was generated. The number of renin-positive granules that contained pixels colocalizing with SNAP23 was counted manually or using segmentation software. Results are expressed as percentage of renin granules showing colocalization with SNAP23. This analysis was performed in multiple cells from two independent preparations.

**Stimulation of renin release.** JG cells were serum deprived for 2 h by replacing the medium with serum-free DMEM (DMEM-SF) containing 100 U/ml penicillin, 100 μg/ml streptomycin, and 5% fetal calf serum. For transient transfections, MS1 cells were seeded in six-well plates at a density of 0.3 × 10^5 cells/well. After 24 h in culture, cells were transfected with 80 nM oligonucleotides plus Lipofectamine 2000 (Invitrogen) following the manufacturer’s instructions. After 48-h posttransfection, cells were lysed, 1.25 μg protein was resolved by SDS-PAGE on 12% polyacrylamide gels, and SNAP23 protein expression was detected using an antibody from Synaptic Systems (1/15,000). As an internal loading control, membranes were reblotted with an antibody against GAPDH (Millipore).

Dominant-negative or shRNA SNAP23 was delivered to primary cultures of mouse JG cells by incubating them with DMEM-SF containing adenoviral particles (100 PFU/cell). After 3 h, fetal calf serum to reach a 5% concentration was added for 24 h in the case of Ad-dn-SNAP23 and 28 h for Ad-si-SNAP23. JG cells were then stimulated with F/IBMX for 1 h as described above.

**Cleavage of SNAP23 with butyltinum neurotoxin E.** Butyltinum neurotoxin E (BotE) cleaves the COOH-terminal portion of mouse SNAP23 (Ad-si-SNAP23), an oligonucleotide fragment encoding 19 nucleotides (nt) of mouse sense SNAP23 (sequence #4) followed by a loop region (TTCAGAGA) and the antisense of the 19 nt was subcloned into the 5′ AflII and 3′ SpeI sites of the adenovector pMIGHTY (Viraquest, North Liberty, IA). Oligonucleotides encoded AflII and SpeI sites at the 5′ and 3′ ends, respectively, for easier insertion into the adenovector. The control construct (Ad-si-Cont) was generated similarly using a scrambled sequence (5′-TTTCCGCAA-CGTGTCACGT-3′). Constructs were sequenced before production of viral particles.

**Reagents.** Fetal calf serum was obtained from Hyclone, and DMEM culture medium and antibiotics from Invitrogen. Forskolin, IBMX, Percoll, and protease inhibitors were from Sigma. BotE was from Metabolics (Madison, WI). Poly-d-lysine from Millipore and the RIA kits used to measure ANG I from Diacor (Stillwater, MN).

**Concentration of adenoviral vectors.** Adenovirus-cytomegalovirus (AdCMV)-hemagglutinin (HA)-tagged SNAP-23AC8 (Ad-dn-SNAP23) was generated as reported previously (5, 21). For construction of adenoviral particles encoding short hairpin silencing RNA (shRNA) against SNAP23, we first tested four separated double-strand node oligonucleotide sequences (sequences #1–4) against mouse SNAP23. Sequence information was obtained from SA Biosciences and oligonucleotide sense and antisense synthesized by Eurofins MWG Operon: sequence #1: 5′-ACAACUCCACCAUGAGUGUTT-3′, sequence #2: 5′-AGGAGAUUUCCUCAAGGUUTT-3′, sequence #3: 5′-AGGUUCUGGAUCAGUUUTT-3′, and sequence #4: 5′-GCUUCUGUGAAU-AAAUTT-3′.
Statistical analysis. Data were expressed as means ± SE and subjected to statistical analysis by t-test with correction of the rejection level using Hochberg’s method or one-way ANOVA with multiple comparisons made by the Student-Newman-Keuls method. A value of P < 0.05 was considered significant.

RESULTS

SNAP23 is expressed in renin-containing secretory granules in primary cultures of mouse JG cells. Consistent with our recent report showing SNAP23 mRNA expression in JG cells (23), we show that SNAP23 protein is abundantly expressed in primary cultures of JG cells. By Western blot, we detect a band corresponding to the predicted molecular weight of 23 kDa similar to that in brain homogenate used as a positive control (Fig. 1A; n = 4). Although SNAP25 is mainly neuronal, it is expressed in other endocrine organs and plays a role in the regulated exocytic pathway (16). We found that SNAP25 is not detectable in JG cells. However, we can detect a clear band at the expected molecular weight in a brain homogenate used as a positive control (Fig. 1B).

Most importantly, we determined the subcellular localization of SNAP23 in JG cells by immunofluorescence and confocal microscopy. Double immunofluorescence labeling of JG cells with antibodies for renin (green) and SNAP23 (red) showed abundance of SNAP23 in renin-containing large secretory granules. Quantitative analysis of colocalizing granules revealed that 84 ± 4% of renin-labeled granules was also positive for SNAP23 (Fig. 1C).

We then proceeded to unequivocally demonstrate the function of SNAP23 in renin release employing several strategies.

Dominant-negative SNAP23 mutant protein partially blocks cAMP-stimulated renin release in primary cultures of mouse JG cells. The first strategy we employed to test whether SNAP23 is involved in cAMP-stimulated renin release is by a dominant-negative approach. We previously showed that transduction of a COOH terminus (8 aa) truncated SNAP23-inhib-
To ensure the efficacy of sequence #4 in JG cells, we then subcloned sequence #4 into an adenovector and viral particles were produced and tested. Transduction of JG cells for 28 h with adenovirus silencing SNAP23 (Ad-si-SNAP23) resulted in a ~50% reduction in SNAP23 protein compared with adenovirus-scrambled sequence (Ad-si-Cont; n = 3; P < 0.05) without affecting VAMP2, VAMP3, or VAMP4 expression levels (Fig. 4B).

Adenoviral-mediated delivery of silencing-SNAP23 partially blocks cAMP-stimulated renin release in primary cultures of mouse JG cells. We then employed a third and last approach to demonstrate the involvement of SNAP23 on cAMP-stimulated renin release by knockdown expression of endogenous SNAP23 using the Ad-si-SNAP23. We found that in JG cells transduced with a scrambled sequence (Ad-si-Cont) cAMP stimulated renin release from 1.07 ± 0.12 to 2.82 ± 0.43% of renin content. However, in JG cells transduced with adenovirus silencing SNAP23 (Ad-si-SNAP-23), cAMP-stimulated renin release was impaired by ~55% (from 1.13 ± 0.22 to 1.9 ± 0.22% of renin content; Fig. 5A). Total renin content from silencing SNAP23 was unaffected compared with the scrambled-transduced group (P = N.S.; n = 7; Fig. 5B), indicating that SNAP23 is not likely involved in renin-containing granule maturation. These results taken together indicate that SNAP23 is implicated in stimulated renin release.

**DISCUSSION**

Renin is the rate-limiting enzyme in the generation of ANG II, which has been long-lasting identified and targeted to regulate blood pressure (reviewed in Ref. 10). In addition, a detrimental role of renin independent of its enzymatic activity (18) has renewed an interest in directly targeting renin per se. It would seem even more attractive and efficient to prevent the release of renin by targeting the proteins involved in its exocytosis. However, fundamental questions on how renin is released from JG cells and the proteins and molecular mechanisms involved have not been explored to date.

Since renin is stored in large dense core granules in JG cells (10) and undergo exocytosis (4, 10, 23, 26, 33), these secretory granules very likely employ the SNARE membrane fusion machinery (2). In fact, we just identified VAMP2 to be one of the three minimally required SNARE family members that mediate cAMP-stimulated renin release in JG cells (23). However, the SNAP isoform mediating renin release is not known. The current work identified the second putative SNARE protein showing that SNAP23, and not SNAP25, is expressed in JG cells, we then employed a third and last approach to demonstrate the involvement of SNAP23 on cAMP-stimulated renin release by knockdown expression of endogenous SNAP23 using the Ad-si-SNAP23. We found that in JG cells transduced with a scrambled sequence (Ad-si-Cont) cAMP stimulated renin release from 1.07 ± 0.12 to 2.82 ± 0.43% of renin content. However, in JG cells transduced with adenovirus silencing SNAP23 (Ad-si-SNAP-23), cAMP-stimulated renin release was impaired by ~55% (from 1.13 ± 0.22 to 1.9 ± 0.22% of renin content; Fig. 5A). Total renin content from silencing SNAP23 was unaffected compared with the scrambled-transduced group (P = N.S.; n = 7; Fig. 5B), indicating that SNAP23 is not likely involved in renin-containing granule maturation. These results taken together indicate that SNAP23 is implicated in stimulated renin release.

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Our immunofluorescence labeling and confocal imaging showed that most (84%) renin-containing dense core granules also contained SNAP23. However, we also observed that some granules containing renin did not express SNAP23. The antibody used to label renin recognizes both, renin and its proform prorenin. Thus, it is possible that renin-labeled granules that colocalized with SNAP23 might contain the mature/active form of renin, whereas those granules that do not possess SNAP23 presumably contain the immature proform, prorenin. Although speculative, this might point to SNAP23 being involved in a specific step of granule fusion (presumably with the plasma membrane) rather than an involvement in renin maturation. Our results are in

![Graph](image-url)
agreement with those of others that demonstrated that SNAP23 is expressed not only at the plasma membrane but in vesicle and large granule membranes (16, 20).

Employing three strategies, we found that SNAP23 mediates cAMP-stimulated renin release in primary cultures of mouse JG cells. Our results show that blockade of renin release is partial after shRNA reduction of SNAP23 expression or proteolytic destruction with BotE, but nonetheless, the reduced secretion (≈50%) was proportionate to the reduction in endogenous levels of SNAP23 (≈50%). Although it is desirable to have complete ablation of SNAP23 expression, these results strongly suggest that SNAP23 is the dominant SNAP isoform that mediates cAMP-evoked renin release. Attempts to increase reduction of SNAP23 levels beyond ≈50% unfortunately resulted in cell damage, observed morphologically and also by a higher basal renin release. Another strategy would be by SNAP23 genetic deletion, but such SNAP23-null mice were recently reported to be not viable (32). We found that silencing or ablating SNAP23 did not affect the amount of total active renin in JG cells (renin content), suggesting a primary role of SNAP23 in the final steps that mediate renin release from granules rather than in renin maturation or the regulation of the total pool of renin.

In brain and neuroendocrine pancreatic islet beta cell exocytosis, SNAP25 is the main isoform mediating exocytosis (12), whereas SNAP23 is the major isoform in most nonneuronal secretory cells, such as pancreatic exocrine cells (16). Consistently, SNAP23 but not SNAP25 is expressed in nonneuronal JG cells. As SNAP25 could be functionally redundant to SNAP23 (28), the absence of SNAP25 in JG cells could explain the toxic effects of knocking down SNAP23 protein since SNAP25 cannot compensate for the lack of SNAP23. Botulinum neurotoxins have been widely used to identify the SNAREs mediating vesicle fusion in many mammalian cells studied (19) and exhibit target specificity. Although BotE can specifically cleave murine SNAP23 and SNAP25 (34, 35), we found that SNAP25 is not expressed in JG cells. Thus, it is unlikely that the blockade of renin release by BotE is due to SNAP25 cleavage.

The SNAP23 homolog SNAP29 has also been described to be ubiquitously expressed in mammalian cells (13). However, SNAP29 has been shown to preferentially bind to syntaxin isoforms involved in endosomal and trans-Golgi fusion events (13) and is expressed almost exclusively in organelles mediating postendocytic trafficking, whereas SNAP23 has been shown to mediate the ultimate step in regulated granule fusion at the plasma membrane in nonneuronal secretory cells (9, 27). Much less is known about the role of the isoform SNAP47 (14). While our data demonstrate that SNAP23 mediates most
SNAP23 MEDIATES cAMP-STIMULATED RENIN RELEASE

Fig. 4. Knockdown of SNAP23 protein expression by adenoviral delivery of short hairpin silencing in JG cells. A: noncoding (lane 2) and 4 SNAP23 double-strand oligonucleotide sequences (lanes 3–6) were transfected into mouse cell line (MS1) cells with liposomes as described in MATERIALS AND METHODS. Forty-eight-hour posttransfection, cells were lysed, and their efficiency was tested by Western blot using SNAP23 antibody. Lane 1 shows an MS1 lysate without any treatment. B: sequence #4 was subcloned in adenovector and viral particles were tested in JG cells. JG cells were transduced with a scrambled sequence (lane 1) or silencing SNAP23 adenovirus (lane 2). After 28-h incubation, JG cell lysates were lysed and resolved in 12% SDS-PAGE for immunoblotting for detection of SNAP23 knockdown efficiency. Membranes were blotted for GAPDH as loading control. Knockdown of SNAP23 did not affect VAMP2, VAMP3, and VAMP4 protein levels. C: optical density quantitation from SNAP23. Band was normalized to GAPDH band in each experiment, and the means ± SE of at least 4 independent experiments were determined (*P < 0.05).

Fig. 5. Silencing SNAP23 blocks cAMP-stimulated renin release in mouse JG cells. A: renin release. After transduction of JG cells for 28 h, cells were serum starved for 2 h and treated for 1 h with F/IIBMX (10 μM/0.5 mM) or vehicle according to the description in MATERIALS AND METHODS. Black bars are JG cells transduced with a scrambled sequence (Ad-si-Cont). Gray bars are JG cells transduced with silencing SNAP23 adenovirus (Ad-si-SNAP23; n = 7; #P < 0.01 vs. vehicle-treated Ad-si-Cont; ∗P < 0.03 vs. Ad-si-Cont + F/IIBMX). B: total renin. Total renin content values are corrected by protein concentration (ng ANG I h incubation/νg protein). Renin content from Ad-si-Cont vehicle-treated was arbitrarily set to 100. Data are expressed as means ± SE (n = 7; P = N.S.).

The precise mechanisms that mediate renin release have not been fully elucidated. Our previous and current work demonstrated that fusion proteins, namely VAMP2 (23) and SNAP23, are involved in stimulated renin release. Future studies will be directed at identifying the cognate syntaxin(s). It is possible that deregulated renin secretion causing hypertension could be enhanced by pathologic coupling of cAMP signaling to this exocytic complex via SNAP23 or that this fusion complex assembly is itself perturbed. Thus, the JG renin granule exocytic complex might present a provocative alternative target for drug development to control blood pressure.

GRANTS

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