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Endothelin-Converting Enzyme-1 Degrades Internalized Somatostatin-14

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Agonist-induced internalization of somatostatin receptors (sst) determines subsequent cellular responsiveness to peptide agonists and influences sst receptor scintigraphy. To investigate sst2A trafficking, rat sst2A tagged with epitope was expressed in human embryonic kidney cells and tracked by antibody labeling. Confocal microscopical analysis revealed that stimulation with sst and octreotide induced internalization of sst2A. Internalized sst2A remained sequestered within early endosomes, and 60 min after stimulation, internalized sst2A still colocalized with ECE-1, calcitonin receptor-like receptor (CLR), and rab5a. Internalized 125I-Tyr1-SST-14 was rapidly hydrolyzed by endosomal endopeptidases, with radioactive metabolites being released from the cell. Internalized 125I-Tyr1-octreotide accumulated as an intact peptide and was released from the cell as an intact peptide ligand. We have identified ECE-1 as one of the endopeptidases responsible for inactivation of internalized SST-14. ECE-1-mediated cleavage of SST-14 was inhibited by the specific ECE-1 inhibitor, SM-19712, and by preventing acidification of endosomes using bafilomycin A1, ECE-1 cleaved SST-14 but not octreotide in an acidic environment. The metallopeptidases angiotensin-1 converting enzyme and ECE-2 did not hydrolyze SST-14 or octreotide. Our results show for the first time that stimulation with SST-14 and octreotide induced sequestration of sst2A into early endosomes and that endocytosed SST-14 is degraded by endopeptidases located in early endosomes. Furthermore, octreotide was not degraded by endosomal peptidases and was released as an intact peptide. This mechanism may explain functional differences between octreotide and SST-14 after sst2A stimulation. Moreover, further investigation of endopeptidase-regulated trafficking of neuropeptides may result in novel concepts of neuropeptide receptor inactivation in cancer diagnosis. (Endocrinology 149: 2200–2207, 2008)
the calcitonin gene-related peptide (CGRP) (CRL) (11). ECE-1 is a metalloendopeptidase related to nephrilysin (12). Its four isoforms (ECE-1a–d) share a catalytic domain, but differences in the cytosolic tail determine their subcellular location (13–15). All four isoforms are present within vesicles inside the cell, and ECE-1a as well as ECE-1c are also present at the plasma membrane. We observed that ECE-1 degrades and inactives SP and CGRP. Endosomal degradation of ligands by ECE-1 allowed recycling and sensitization of the receptors. These experiments identified a new role for ECE-1 in regulating receptor trafficking.

Recent studies of sst2A have shown that the β-arrestin-dependent trafficking of the sst2A sst receptor resembled that of a “class B” receptor (6). Furthermore, investigation of dependent trafficking of the sst2A sst receptor resembled concentration of the receptor within early endosomes. Internalized 125I-SST-14 was not routed to lysosomes for degradation of the receptor within early endosomes. Internalized 125I-Tyr11-SST-14 is degraded by endosomally located peptidases. Interestingly, inhibition of the activity of the endosomally located peptidases ECE-1 with SM-19712 or preventing acidification of endosomes using bafilomycin A1 only partially inhibits degradation of SST-14, indicating that other peptidases participate in the degradation of internalized SST-14. Two iodinated metabolites of SST-14 accumulated within the cell during stimulation, whereas mainly one peptide metabolite was detected in the supernatant. Internalized 125I-Tyr11-SST-14 was not routed to lysosomes for degradation. Interestingly, octreotide accumulated as an intact peptide in the cells and was slowly released as an intact peptide from the cells. Further investigation of the metalloendopeptidases mediating degradation of sst showed that SST-14 was not degraded at acidic pH or neutral pH by recombinant human (rh) ECE-2 and rh angiotensin-1 converting enzyme (ACE-1), whereas ECE-1 just degraded SST-14 at an acidic pH. In contrast, octreotide was not degraded by rhECE-1, rhECE-2, or rhACE.

Materials and Methods

Materials

SST-14 and octreotide were from Bachem (Torrance, CA). 125I-Tyr11-SST-14 was from Amersham Biosciences (Braunschweig, Germany), and 125I-Tyr1-octreotide was from Anawa (Cologne, Germany). Mouse antimouse coupled to fluorescein isothiocyanate or Texas Red was from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). Biotinylated goat anti-ECE-1 antibody, rhACE, rhECE-1, and rhECE-2 were from R&D Systems, Inc. (Minneapolis, MN). The ECE-1 substrate McaBK2 [(7-methoxyxocoumarin-4-v)-acyctyl-Arg-Pro-Pro-Gly-Phe-Ser-Ala-Phe-Lys(2A-dinitrophenyl)]. ECE-1 inhibitor SM-19712 (4-chloro-1-N-[[[(2-cyano-3-methyl-1-phenyl-1H-pyrazol-5-ylamino]carbonyl] benzenesulfonyamide, monosodium salt), and bafilomycin A were from Sigma Chemical Co. (St. Louis, MO). Lipofectamine 2000 was from Invitrogen (Karlsruhe, Germany). Media, sera, and plastics were from Life Technologies, Inc./BRL (Eggenstein, Germany). Other reagents were from Sigma Chemical. The plasmid construct of rat sst2A containing the amino-terminal T7 epitope tag sequence MASMTGGQQMG in pcDNA3.1 has been described previously (6). Constructs for β-arrestin1-ECE-1GFP and rab5a-EGFP were generated as described (16, 17).

Cell culture and transfection

Human embryonic kidney (HEK) 293 cells were grown in DMEM supplemented with 10% fetal calf serum in a humidified atmosphere containing 10% CO2. Cells were transfected using Lipofectamine 2000 according to the manufacturer’s instructions. HEK293 cells stably expressing T7-sst2A were selected in the presence of 400 μg/ml G418 (Invitrogen).

Microscopy and immunofluorescence

Cells were incubated in DMEM plus 0.1% BSA with 100 nm SST-14 or octreotide for 10 min at 37°C, washed, and incubated for 0–60 min at 37°C. Cells were fixed with 4% paraformaldehyde in 100 mM PBS (pH 7.4) for 20 min. Cells were incubated for 30 min in Hanks’ buffered saline solution (HBSS), 5% normal goat serum, and 0.1% saponin. The sst2A was detected using mouse T7 antibody (1:10,000, overnight at 4°C) and Texas Red-conjugated goat antimouse IgG (1:200, 2 h, room temperature). β-Arrestin1 and rab5a were detected using EGF. ECE-1 was detected using biotinylated goat anti-ECE-1 antibody (1:400) and fluorescein isothiocyanate-coupled streptavidin (1:500). Cells were observed using a 510 Meta confocal microscope (Zeiss, Germany) with a Plan Apo x63 (numerical aperture 1.4) objective.

Binding assays

Cells grown in 24-well dishes were stimulated with SST-14 (10 nm) or octreotide (100 nm) in DMEM (0.1% BSA) for 0–10 min at 37°C. Cells were washed once with warmed acid buffer (HBSS-acetic acid buffer (pH 4.8)) and once with HBSS (0.1% BSA). Cells were incubated for 0–120 min at 37°C, placed on ice, and incubated with 100,000 cpm 125I-Tyr11-SST-14 for 120 min at 4°C. Unbound 125I-Tyr11-SST-14 was washed off with HBSS (0.1% BSA). Cells were lysed in 1 ml NaOH, and bound 125I-Tyr11-SST-14 was collected and determined in a γ-counter (Canberra Packard, Dreieich, Germany) (18).

HPLC analysis of 125I-Tyr11-SST-14 and 125I-Tyr1-octreotide

Cells were incubated with 100,000 cpm/0.35 ml 25I-Tyr11-SST-14 or 125I-Tyr1-octreotide mixed with 1 pm unlabeled peptide in HBSS (0.1% BSA) for 0–15 min at 37°C. After 15-min stimulation, cells were washed once with warmed acid buffer (pH 4.8) (18). In some experiments the cells were preincubated with SM-19712 (10 μM) or bafilomycin A1 (1 μM) for 30 min. Cells were washed once with HBSS (0.1% BSA), incubated for 15 min at 37°C in 0.5 ml HBSS (0.1% BSA), washed, and incubated for 60 min. The supernatants were collected, acidified by adding 50 μl trifluoroacetic acid (TFA) (10%), and centrifuged (5 min, 13,000 g). Cell lysates were acidified with 0.5 ml 0.08% TFA and centrifuged (5 min, 13,000 g). Samples were fractionated by reversed-phase HPLC using a C-18 column (2 × 25 mm). A separating gradient of 0–40% acetonitrile, 0.08% TFA, 25 min, 1 ml/min was used (HPLC-Model Akta purifier; General Healthcare, Munich, Germany). HPLC fractions were collected every minute and counted (4, 19, 20).

rhACE, rhECE-2, and rhECE-1 enzymatic activity

Peptidase activity was measured using McaBK2 (21). McaBK2 (6 μM) was incubated with 0.5 μg rhACE, rhECE-2, or rhECE-1 in 50 mm 2-(N-morpholino)ethanesulfonic acid (pH 5.5) or 50 mm Tris/HCl (pH 7.4) at 37°C. Fluorescence was measured at λ ex 320 nm and λ em 405 nm.

Peptide degradation by rhACE, rhECE-2, and rhECE-1

SST-14 or octreotide (250 μM) was incubated with rhACE, rhECE-2, or rhECE-1 (0.5 μg) in 150 μl of 50 mm (2-N-morpholino)ethanesulfonic acid (pH 5.8) or 50 mm Tris/HCl (pH 7.4) for 0–90 min at 37°C. Reactions were stopped by adding 50 μl of 20% TCA, and peptide degradation was measured by HPLC analysis.
were stopped by boiling for 3 min. Reaction products were separated by reversed-phase HPLC using a C-18 column equilibrated in 0.1% TFA in water, and eluted using a linear gradient of 0.1% TFA in acetonitrile (0–40% acetonitrile, 25 min) at 1 ml/min. Peptides were detected by spectrophotometry (A256 nm). Products were collected and analyzed by time-of-flight mass spectrometry.

Results

Agonists of sst2A induce receptor internalization and association with β-arrestin1

“Class B” receptors form stable and high-affinity interactions with β-arrestin1 and β-arrestin2 at the cell surface and in endosomes (7, 22). To further characterize the internalization of rat somatostatin receptor 2A (rsst2A), we examined the colocalization of β-arrestin1 with internalized rsst2A. A fusion protein of β-arrestin1 and EGFP (β-arrestin1-EGFP) and T7-rsst2A were transiently coexpressed in HEK293 cells (23). In unstimulated cells, β-arrestin1-EGFP was evenly distributed in the cytoplasm and nucleus (Fig. 1). Stimulation with SST-14 or octreotide for 10 min induced translocation of β-arrestin1-EGFP from the cytosol to the cell membrane, where it colocalized with sst2A. At later times SST-14 and octreotide induced endocytosis of sst2A and β-arrestin1-EGFP to small vesicles close to the cell membrane. The distribution and size of the vesicles are similar to those found in studies analyzing sst2A-mediated internalization of fluorescent SST-14 (24). Internalized sst2A then translocated to hollow core vesicles that also contained β-arrestin1-EGFP (Fig. 1, A and B, arrowheads). Interestingly, in cells stimulated with SST-14, β-arrestin1 is associated with internalized rsst2A (Fig. 1, A and B, arrowheads) and at the cell membrane (Fig. 1, A and B, arrows). In cells stimulated with octreotide, β-arrestin1 is mainly associated with internalized rsst2A. The fluorescence intensity scan (Fig. 1, A and B, yellow arrows) of the cells is shown in Fig. 1, E and F. The fluorescence signal of β-arrestin1-EGFP is more intensified at the plasma membrane in cells stimulated with SST-14 (Fig. 1, E and F, green arrows) than in cells stimulated with octreotide. Thus, stimulation of rsst2A with agonist results in translocation of β-arrestin1 to the plasma membrane and subsequent association with rsst2A for prolonged periods indicative of a “class B” GPCR.

Fig. 1. Subcellular distribution of T7-rsst2A. HEK293 cells were transiently transfected with T7-rsst2A, β-arrestin1-EGFP (A and B) or rab5a-EGFP (C). Cells were stimulated with 10 nM SST-14 or 10 nM octreotide, washed, and incubated for 15 or 60 min at 37°C. T7-rsst2A was detected using a mouse anti-T7 antibody and Texas Red-conjugated antimouse antibody. β-Arrestin1-EGFP and rab5a-EGFP were detected with EGFP. ECE-1 was detected using goat anti-ECE-1 antibody. In unstimulated cells, T7-rsst2A was detected at the cell surface. β-Arrestin1-EGFP was detected as a diffuse protein located within the cytosol. Stimulation with the peptide ligand SST-14 for 10 min induced receptor internalization. A and B, Internalized T7-rsst2A was strongly associated with β-arrestin1. Incubation for 15 or 60 min induced the formation of hollow core vesicles (arrowheads). β-Arrestin remained strongly associated with internalized T7-rsst2A even 60 min after cell stimulation. In cells stimulated with SST-14, β-arrestin1 is also located at the cell membrane (arrows). The yellow arrows show the area of the intensity scans. C, Colocalization of internalized T7-rsst2A with rab5a-EGFP 60 min after cell stimulation with SST-14 (10 nM), indicating the localization of internalized T7-rsst2A in early endosomes. D, Colocalization of internalized T7-rsst2A with endogenously expressed ECE-1 60 min after stimulation of the cells with SST-14 (10 nM). Fluorescence intensity scan of cells stimulated with SST-14 (E) and octreotide (F) after 60 min recovery. The fluorescence signal of β-arrestin1-EGFP is more intensified at the plasma membrane (green arrows) in cells stimulated with SST-14 than in cells stimulated with octreotide.
Internalized rsst2A is sequestered in early endosomes

After stimulation with agonist, “class B” GPCRs such as NK₁R, CLR, and angiotensin II type 1A receptor are sequestered within early endosomes for prolonged periods (9, 25, 26). To determine the subcellular location of internalized rsst2A, HEK293 cells were transiently transfected with T7-rsst2A and a marker protein for early endosomes, rab5a-EGFP, and their localization was examined using immunofluorescence and confocal microscopy. In unstimulated cells, rsst2A was present at the plasma membrane, and rab5a was present in vesicles within the cell (data not shown). After stimulation with SST-14 (10 nm, 10 min) and incubation in SST-14-free medium for 60 min, internalized rsst2A colocalized with rab5a-EGFP in small and hollow core vesicles (Fig. 1C, arrowheads). Thus, after stimulation with SST-14, rsst2A traffics to early endosomes and remained there for at least 60 min after stimulation.

Internalized rsst2A colocalizes with endogenous ECE-1

HEK293 cells endogenously express all four isoforms of ECE-1 (10). Within the acidic environment of early endosomes, ECE-1 has inactivated the peptide ligands of some “class B” GPCRs (10, 11). We have previously demonstrated that SP and CGRP are inactivated by ECE-1 endogenously expressed by HEK293 cells. HEK293 cells were transiently transfected with rsst2A and the receptor and ECE-1 localized by immunofluorescence and confocal microscopy. In unstimulated cells, rsst2A was localized at the plasma membrane, and ECE-1 was distributed in cytoplasmic vesicles. After stimulation rsst2A was localized at the plasma membrane and colocalized with ECE-1 in vesicles. Thus, rsst2A is transported from the plasma membrane to early endosomes where it remains colocalized with ECE-1 (Fig. 1D).

Recovery of agonist-binding sites after rsst2A stimulation

Another characteristic of “class B” GPCRs is that they slowly recycle back to the cell surface because they were sequestered within early endosomes. Confocal microscopical analysis of rsst2A expressed in rat insulinoma cell line 1046–38 or in HEK293 cells suggests that rsst2A recycled to the plasma membrane within 1–2 h (6, 18). Therefore, we analyzed rsst2A recycling by determining the reappearance of cell surface binding sites after stimulation with SST-14 or octreotide. Rat rsst2A-HEK293 cells were stimulated with SST-14 or octreotide, and the recovery of surface binding sites was determined by incubation with 125I-SST-14. We found that stimulation with SST-14 or octreotide rapidly induced a marked reduction of surface binding sites (Fig. 2). After stimulation (10 min, 37°C), surface binding sites of SST-14 (51 ± 3%) and octreotide (45 ± 4%) were markedly reduced. Interestingly, the binding sites did not recover during 2-h incubation at 37°C. These findings indicate that rsst2A remained internalized for at least 2 h after stimulation with both peptide ligands. This slow recovery of binding sites is indicative of the classical trafficking of a class B GPCR.

Internalized 125I-Tyr₁¹·SST-14 but not 125I-Tyr₁·octreotide is degraded by endosomal endopeptidases

Because the receptor-ligand complex of “class B” receptors is sequestered within acidic early endosomes, endosomal located peptidases are capable of mediating the degradation of the endocytosed peptide ligands (10, 11). We analyzed the degradation of 125I-SST-14 and 125I-octreotide in HEK293 cells stably expressing rsst2A. Cells were incubated for 0–15 min with 100,000 cpm radiolabeled peptide, mixed with 1 nM unlabeled peptide in HBSS (0.1% BSA) at 37°C, washed twice with acidic HBSS (pH 4.8), and once with HBSS (0.1% BSA), and incubated for 0–90 min at 37°C. Cell lysates and supernatants were collected and analyzed by HPLC. Analysis of cell lysates from SST-14-treated cells indicates that SST-14 was rapidly endocytosed by rsst2A-HEK293 cells (Fig. 3A, closed diamonds). SST-14 was rapidly degraded into two radioactive labeled peptide metabolites that accumulated within the cell. After 15-min incubation with 125I-SST-14, only 42.9% of total radioactivity was found to be cell associated as intact 125I-SST-14. Of the remaining radioactivity, 19.3% was peptide A (retention time, 9 min), and 37.7% was peptide B (retention time, 11 min) (Fig. 3A). Incubation for longer periods at 37°C induced further degradation of the internalized SST-14. At 90 min after stimulation, only 7.3% of the total radioactivity remained as intact SST-14, whereas 13.7% of the total radioactivity was peptide B, and 2.6% of the total radioactivity was peptide A. Interestingly, most of the radioactivity was released from the cells (Fig. 3C). HPLC analysis of supernatants demonstrated that two peptide metabolites were released from the cells. The major product was peptide B (69.2% of the total radioactivity), with the minor product, peptide A, only being 4.9% of the total radioactivity. The small amount of peptide A detected into the supernatant (4.9%, even after 90 min) is in marked contrast to the maximal amount associated with the cell after 15 min (19.3% of total radioactivity). These findings suggest that peptide A is an intermediate degradation product before being further processed to peptide B.

We found that incubation of rsst2A cells with 125I-octreotide
induced accumulation of intact octreotide in the cells (Fig. 3B). Ninety minutes after cells were washed, 83% of the internalized octreotide was still cell associated, and 17% of the radioactivity was found in the supernatant as intact octreotide (Fig. 3D).

As shown in a representative HPLC chromatogram (Fig. 3E), intact 125I-Tyr11-SST-14 eluted by 16 min, and two radioactive degradation products were recognized by HPLC as having eluted by 9 and 11 min, respectively. The 225Tyr eluted by 3–4 min and could not be detected in the cell lysate or the supernatant of rsst2A-HEK cells, indicating that SST-14 was not routed to lysosomal degradation (11).

HEK293 cells endogenously express all four isoforms of ECE-1 (10). Therefore, in our next set of experiments, we tried to inhibit intracellular degradation of internalized 125I-SST-14 by preincubating the cells with the ECE-1-specific inhibitor, SM-19712 (10 μM), and by neutralizing acidic vesicles by preincubating with bafilomycin A1 (1 μM). Both inhibitors had been previously tested for the ECE-1-mediated degradation of internalized SP and CGRP (10, 11). We preincubated cells for 15 min with 100,000 cpm 125I-Tyr11-SST-14 mixed with 1 nM SST-14 in the presence of the inhibitors, washed, and incubated for 60 min at 37°C. Without inhibitor, 7.3% (4) of the total radioactivity was intact 125I-Tyr11-SST-14. In cells pretreated with SM-19712, 24% of the total radioactivity was intact 125I-Tyr11-SST-14. In cells pretreated with bafilomycin A, 26% of the total radioactivity was intact 125I-Tyr11-SST-14 (\(*\), \(P < 0.01\) to control). G, The sequences of SST-14 and octreotide. The peptides were digested with rhECE-1, and the products were purified by HPLC and identified by mass spectrometry.
other endopeptidases participate in the endosomal degradation of SST-14. Figure 3G shows the sequences of SST-14 and octreotide. The arrows mark the sites of cleavage by ECE-1 inferred from the product masses.

We next sought to determine whether other endopeptidase were able to degrade SST-14. Therefore, we tested rhECE-1, rhECE-2, and rhACE-1 to see if they degraded SST-14 and octreotide. The activity of the metalloendopeptidases was measured with the synthetic fluorescence substrate McaBK2. We tested the degradation of McaBK2 at neutral pH and at pH 5.8, which is equal to the pH of acidic early endosomes (10). The metalloendopeptidases are active at pH 5.8 (data not shown). At neutral pH, rhACE degrades McaBK2 10 times faster than at pH 5.5, whereas rhECE-2 did not degrade McaBK2 at pH 7.4 (data not shown). While testing the degradation of SST-14 and octreotide, we found that ECE-1 did not degrade octreotide in an acidic environment but did degrade SST-14 (Fig. 4, A and B). Time-of-flight mass spectroscopy showed that SST-14 was degraded at position Asn5-Phe6 and at position Thr10-Phe11, leading to two degradation products: the peptide SST-14 (1–5)–SST-14 (11–14) and SST-14 (6–10).

Figure 4C shows the time course of rhECE-1 mediated degradation of SST-14 in an acidic and neutral environment. At pH 5.8, rhECE-1 rapidly degraded SST-14. After 30-min incubation, 50% of the SST-14 was degraded. Interestingly, prolonged incubation with rhECE-1 did not result in a complete degradation of the peptide. A similar incomplete degradation profile was observed for the ECE-1 mediated degradation of CGRP (11).

Figure 4D shows the amount of intact peptide for octreotide and SST-14 after 90-min incubation with rhACE, rhECE-2, and rhECE-1. Of note, octreotide was not degraded by any of these endopeptidases. SST-14 was only degraded by ECE-1 in an acidic environment by approximately 50% (Fig. 4D). None of the other tested endopeptidases hydrolyzed SST-14.

**Discussion**

The first aim of this investigation was to determine the subcellular localization of internalized sst2A. Our results show that stimulation with SST-14 or octreotide induced internalization of sst2A into early endosomes. Even 60 min after stimulation, sst2A still colocalized with the early endosomal marker protein rab5a. These results, including the strong association of internalized rsst2A with β-arrestin1–EGFP, indicate that sst2A trafficking resembles the trafficking mechanism of a “class B” receptor (9, 26). Interestingly, β-arrestin1 is located at the cell membrane and at early endosomes when the cells are stimulated with SST-14, whereas β-arrestin1 is mainly associated with early endosomes after stimulation with octreotide. The data let us suggest that degradation of SST-14 allowed dissociation of β-arrestin1 from internalized rsst2A and translocation of β-arrestin1 to the cell membrane. Our results are in line with previous results analyzing β-arrestin trafficking and endosomal sorting of other sst receptors subtypes (6, 18). Interestingly, whereas confocal analysis of the recycling behavior of sst2A in rat insulinoma 1046–38 cells or HEK293 suggests that sst2A recycled within 1–2 h, we were unable to observe recycling of rsst2A 60 min after stimulation. That we were able to determine the reappearance of surface binding sites indicates that sst2A did not recycle 2 h after stimulation with the cognitive ligand.

Another characteristic of “class B” GPCRs is the formation of hollow core vesicles. This has been clearly demonstrated for the AT(1A)R and the NK1R (9, 26). Hollow core vesicles are enlarged vesicles, which are formed by the constitutively active rab5a mutant rab5aQ79L. Other studies found that rab5a is a binding protein of the AT(1A)R and suggested that
an intrinsic guanosine 5'-diphosphate-guanosine 5'-triphosphate (GTP) exchange element is encoded in the carboxy-terminal tail of the AT1A receptor (25, 26). The direct interaction of phosphorylated receptor and activated (GTP-bound) rab5a may lead to the homotypic fusion of early endosomes and the subsequent formation of hollow core vesicles. A detailed analysis of deletion mutants of AT1A demonstrated that truncation of the C-terminal tail of the receptor inhibits the formation of hollow core vesicles but does not inhibit internalization of the receptor (26). Previous studies showing that the guanosine 5'-diphosphate-GTP-exchange element is coded at the far end of the C terminus of the AT1A receptor let us suggest that the naturally occurring splice variant of sst2A, sst2B, demonstrates a different pattern of internalization, resensitization, and trafficking compared with sst2A (27, 28).

Our confocal microscopic observation of T7-rsst2A and β-arrestin1-EGFP also shows that stimulation with SST-14 and octreotide induced the formation of β-arrestin1 and coated hollow core vesicles. These results indicate that rss2A trafficking is blocked in early endosomes. The localization within early endosomes could be also demonstrated by the colocalization of internalized rss2A with rab5a-EGFP. Because the receptor is not immediately transported to late endosomes, and the ligand is not routed into lysosomal degradation, endopeptidases with an acidic pH optimum for degradation of small peptides, like ECE-1 or ECE-2, should be involved in the hydrolysis and inactivation process of the endocytosed peptide ligands (29, 30). Recently, the endopeptidase ACE was also cotransported with internalized receptors (31), suggesting that this degrading enzyme may not necessarily be constitutively located within early endosomes. The degradation of an internalized peptide agonist by early endosomally located endopeptidases was recently demonstrated for the NK1 receptor and for the CLR/CGRP system (10, 11). The same studies showed that for both class B receptors, intracellular ECE-1 inactivates the internalized ligand within early endosomes and allowed dissociation of β-arrestin1 from the internalized receptor. They also showed that the degradation of ligand by ECE-1 is an essential step in the process of neuropetide receptor trafficking and resensitization. In particular, ECE-1 cleaved SP at positions Gln6–Phe7 and Gly9–Leu10 to form the metabolites SP[1–6] and SP[1–9]. Similar to the degradation of SP and CGRP, that of SST-14 by ECE-1 is also sensitive to a specific pH.

The metabolites of ECE-1-mediated degradation of SP, SP[1–6] and SP[1–9], accumulated within the cells and were slowly released into the supernatant. In contrast, the metabolites of CGRP transiently occurred within the cells and were most probably transported toward lysosomal degradation. Here, we demonstrate that the preferred cleavage sites of SST-14 are at positions Asn5–Phe6 and Thr10–Phe11. ECE-1 Here, we demonstrate that the preferred cleavage sites of SST-14 by ECE-1 is also sensitive to a specific pH. Similar to the degradation of SP and CGRP, that of SST-14 is also partially blocked by inhibitors. This observation strongly suggests that other endopeptidases besides ECE-1 participate in the endosomal degradation process of SST-14. Therefore, we tested whether rhECE-2 and rhACE were able to degrade SST-14 and octreotide. Interestingly, both peptidases were unable to degrade SST-14. Alternatively, an endosomal location for cathepsin B has been recently reported (32). Therefore, we tested whether the cathepsin B inhibitor, CA074-ME, was able to prevent endosomal degradation of SST-14. In fact, pretreatment of cells with CA074-ME before SST-14 stimulation did not influence the endosomal degradation of SST-14 (data not shown).

In the present study, we compared the fate of a natural ligand, SST-14, and the longlasting synthetic SST analog octreotide in cells expressing rss2A. Both peptides induced internalization and sequestration of sst2A within early en-
dosomes. In early endosomes, SST-14 was hydrolyzed and inactivated by different endopeptidases. The endopeptidase-generated degradation products were released as peptide fragments from these cells. At the same time, the internalized receptor remained intracellularly located and was not recycled. Furthermore, the synthetic analog (octreotide) remained as an intact ligand for a prolonged time within the cells, where it colocalized with the internalized receptor sst2A. The finding that an internalized ligand of sst2A can be degraded within early endosomes while not routed into lysosomal degradation will help to develop novel radiolabeled sst or other neuropeptide ligand analogs useful for medical diagnostics such as tumor imaging as well as molecular imaging for in vivo models of inflammation (33, 34).

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