Fates of endocytosed somatostatin sst_2 receptors and associated agonists

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Somatostatin agonists are rapidly and efficiently internalized with the somatostatin sst_2 receptor. The fate of internalized agonists and receptors is of critical importance because the rate of ligand recycling back to the cell surface can limit the amount of radioligand accumulated inside the cells, whereas receptor recycling might be of vital importance in providing the cell surface with dephosphorylated, resensitized receptors. Furthermore the accumulation of radioisotope-conjugated somatostatin agonists inside cancer cells resulting from receptor-mediated internalization has been used as a treatment for cancers that overexpress somatostatin receptors. In the present study, radioiodinated agonists at the sst_2 somatostatin receptor were employed to allow quantitative analysis of the fate of endocytosed agonist. After endocytosis, recycling back to the cell surface was the main pathway for both ^{125}I-labelled somatostatin-14 (SRIF-14) and the more stable agonist ^{125}I-labelled cyclo(*N*-Me-Ala-Tyr-D-Trp-Lys-Abu-Phe) (BIM-23027; Abu stands for aminobutyric acid), accounting for 75–85% of internalized ligand when re-endocytosis of radioligand was prevented. We have shown that there is a dynamic cycling of both somatostatin agonist ligands and receptors between the cell surface and internal compartments both during agonist treatment and after surface-bound agonist has been removed, unless steps are taken to prevent the re-activation of receptors by recycled agonist. Internalization leads to increased degradation of ^{125}I-labelled SRIF-14 but not ^{125}I-labelled BIM-23027. The concentration of recycled agonist accumulating in the extracellular medium was sufficient to re-activate the receptor, as measured both by the inhibition of forskolin-stimulated adenylate cyclase and the recovery of surface receptor number after internalization.

INTRODUCTION

The subcellular distribution of receptors and their agonist ligands is determined by their movement along multiple pathways. Agonist binding to cell surface receptors initiates receptor-mediated endocytosis and accumulation of receptor and agonists in endosomes. From there, agonists and receptors can recycle back to the cell surface and/or be routed to lysosomes for degradation. We have previously shown that, for muscarinic receptors, it is the balance between the rates of endocytosis and recycling that determines the steady-state number of surface receptors on treatment with agonist [1]. Similarly, the relative rates of recycling and lysosomal routing determine how quickly the receptors are down-regulated [1]. In the absence of specific targeting signals, the default pathway for internalized membrane proteins is constitutive recycling back to the cell surface; this can take place with almost 100% efficiency [2–5]. Many cell-surface receptor proteins undergo endocytosis but the sorting between the recycling and degradation pathways varies.

Receptors for transferrin and low-density lipoprotein capture the nutrient at the cell surface and endocytose to enable the nutrient to dissociate from the receptor in the acidic environment of the endosomes; the receptors then recycle back to the cell surface [6–8]. Growth factor receptors such as the epidermal growth factor (EGF) receptor show more complex characteristics. The proportion of ligand recycled depends on the dissociation rate of that ligand in the low pH of the endosomes [9]. Transforming growth factor α (TGFα) dissociates more rapidly than human EGF at low pH and correspondingly a greater proportion of TGFα is recycled than human EGF. The proportion of EGF receptors that are recycled depends on whether the receptor is occupied with ligand. The unoccupied receptor is predominantly recycled, whereas the occupied receptor is directed to lysosomes [10]. TGFα is less effective than EGF in causing EGF receptor down-regulation, which might reflect differential sorting of receptors by ligands with different affinities [11]. Furthermore the signalling activity of the receptor is thought to have some bearing on the sorting outcome of the receptors, because kinase-negative mutant EGF receptors are recycled rather than degraded [12] and this might be related to the phosphorylation of proteins involved in trafficking pathways [6]. Studies with the insulin receptor have also shown that receptors that are unable to dissociate ligand are sorted predominantly to lysosomes rather than being recycled [13].

The G-protein-coupled receptors for small neurotransmitters, which have low affinity for their ligands, are predominantly recycled in a constitutive manner (muscarinic acetylcholine receptors [14,15] and β-adrenergic receptors [16]). The situation with the higher-affinity peptide hormone G-protein-coupled receptors is less clear. Many of the peptide hormone receptors are known to recycle (e.g. angiotensin AT_{1A} receptor [17], thyrotropin releasing hormone receptor [18], gastrin-releasing peptide receptor [19], neuromedin-B receptor [20], neurokinin 1 receptor [21], gastrin/cholecystokinin (CCK)-B receptor [22] and glucagon-like peptide 1 receptor [23]). However, the fate of the hormone itself is often unclear. Although somatostatin agonists at the somatostatin sst_2 receptor bind with high affinity in membrane binding assays [24] and their dissociation rate can be enhanced in acid pH [25], the presence of high concentrations of GTP inside the cell leads to the hypothesis that, once inside endosomes, the ligand dissociates rapidly. If this were true, by analogy with growth factor receptors one would expect that somatostatin agonists and their receptors would be predominantly recycled after endocytosis. However, in studies with other fluorescently labelled peptide hormone agonists, fluorescence could be detected in lysosomes, suggesting that the

Abbreviations used: BIM-23027, cyclo(*N*-Me-Ala-Tyr-D-Trp-Lys-Abu-Phe) (Abu is aminobutyric acid); CCK, cholecystokinin; CHO, Chinese hamster ovary; DMEM, Dulbecco's modified Eagle's medium; EGF, epidermal growth factor; sst_2, somatostatin sst_2 receptor; SRIF-14, somatostatin-14; TGFα, transforming growth factor α.

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ligand is degraded [17,22]. In studies of the GLP-1 receptor, ^{125}I-labelled GLP-1 was found to be directed to lysosomes, where it was degraded [23].

Conjugates of peptidase-resistant somatostatin agonists with toxic radioisotopes have been used as a chemotherapy treatment for certain tumours [26,27]. Because the rates of endocytosis and recycling of the ligand are both determinants of the accumulation of radioactivity inside cells, an understanding of the kinetics of ligand trafficking in cells might lead to the design of better ligands. The aim of this study was to examine whether the somatostatin agonists somatostatin-14 (SRIF-14) and cyclo(*N*-Me-Ala-Tyr-D-Trp-Lys-Abu-Phe) (BIM-23027; Abu stands for aminobutyric acid) and the sst_2 receptor expressed in Chinese hamster ovary (CHO) cells were recycled or degraded after receptor-mediated endocytosis. Quantitative information was obtained by the study of two agonist radioligands, the endogenous ligand SRIF-14 and the more peptidase-resistant agonist BIM-23027 to determine whether or not there were any differences in the fate of these ligands after receptor-mediated endocytosis. An N-terminal epitope-tagged form of the receptor was employed to enable the measurement of changes in surface receptor number.

MATERIALS AND METHODS

Tissue culture

CHO cells containing transfected human sst_2 receptors epitope-tagged with a haemagglutinin tag at the N-terminal domain were obtained from Affymax (Palo Alto, CA, U.S.A.). Cells were grown in Dulbecco's modified Eagle's medium (DMEM)/F12 (1:1) medium supplemented with 0.25 mg/ml geneticin and 10% (v/v) fetal bovine serum.

Radioligand internalization studies

Medium was removed from 24-well plates and cells were washed twice with 2 ml of internalization buffer (Hepes-buffered DMEM, pH 7.2). Internalization buffer (0.2 ml) was added to each well and incubated at 37 °C for 10 min. Radioligand (either ^{125}I-labelled BIM-23027 or ^{125}I-labelled SRIF-14, final concentration 0.1 nM; 0.05 ml) was added from a 5-fold concentrated stock and incubated at 37 °C unless stated otherwise. The stock radioligand was prepared in internalization buffer containing 0.2 mg/ml bacitracin. Non-specific internalization was determined by parallel incubations containing 2 μM SRIF-14. Specific internalization typically accounted for 80–90% of total ligand internalized. Internalized ligand accounted for less than 10% of total ligand added. The incubation was terminated by the removal of incubation medium and rapid washing twice with 1 ml of ice-cold acid wash buffer (DMEM buffered with 10 mM Mes, pH 5.0). Cells were incubated in acid wash buffer for 10 min, a time that had previously been shown to remove over 90% of bound ligand [25]. After removal of the acid wash buffer, cells were rinsed twice with 2 ml of acid wash buffer, solubilized in 1 ml of 1% (v/v) Triton X-100 and transferred to vials for quantification of radioactivity in a γ-counter. Cell density was determined by removing cells from the wells with 0.25 ml of PBS containing 5 mM EDTA and counting the cells with a Coulter counter. There were typically 10000–30000 cells per well.

Treatment with inhibitors of internalization

Intact cells in 24-well plates were preincubated with 0.5 M sucrose for 10 min at 37 °C. Cells were depleted of ATP by incubation with 50 nM antimycin and 50 mM 2-deoxyglucose at 37 °C for 30 min before the internalization assay [28,29]. Antimycin and 2-deoxyglucose were contained in all subsequent incubation buffers.

Radioligand recycling

Cells were incubated with 0.1 nM ^{125}I-labelled BIM-23027 or ^{125}I-labelled SRIF-14 for 60 min at 37 °C, then washed thoroughly with pH 5.0 buffer to remove surface-bound ligand as described above. Cells were then warmed to 37 °C with fresh Hepes-buffered DMEM. In some experiments the recycling buffer contained SRIF-14 or BIM-23027 (concentrations as indicated), which was included to prevent the re-uptake of recycled radiolabelled ligand. The external medium was removed to vials for quantification of radioactivity in a γ-counter. In other experiments the external medium was removed and replaced with fresh Hepes-buffered DMEM at 37 °C every 5 min for 60 min. Internalized ligand remaining inside the cells was solubilized in 1% (v/v) Triton X-100, removed to vials and quantified in a γ-counter.

Separation of intact from degraded peptide ligand

Peptides were separated from amino acid degradation products with a method based on that described in [30]. Although a good separation of intact agonist and tyrosine (the major radiolabelled degradation product) was achieved, this technique cannot distinguish between different forms of partly degraded agonist. A Sep-Pak Plus C_{18} minicolumn was washed with 4 ml of solution B [acetonitrile/water/trifluoroacetic acid (900:100:0.5, by vol.)] and then with 4 ml of solution A [water/trifluoroacetic acid (1000:1, v/v)]. The sample was loaded and salts were eluted with 2 ml of solution A (fraction 1). Amino acids were eluted with 4 ml of a mixture of 80% A and 20% B (fraction 2). Intact peptide was eluted with 4 ml of a mixture of 50% A and 50% B followed by 4 ml of 80% A and 20% B (fraction 3). By using radiolabelled standards it was shown that more than 95% of [^{3}H]tyrosine was eluted in the amino acid fraction (fraction 2) and more than 95% of ^{125}I-labelled BIM-23027 was eluted in the intact peptide fraction (fraction 3). A small proportion (10%) of ^{125}I-labelled SRIF-14 was eluted in fraction 1, which might have been accounted for by free iodide; the remainder was eluted in fraction 3.

Measurement of changes in surface receptor number by enzyme-linked immunoassay

Cells were plated at 10^4 cells per well on 24-well plates 24 h before the experiment. The cells were rinsed twice with 1 ml of Hepes-buffered DMEM, pH 7.2, and incubated with agonist at the indicated concentration at 37 °C to induce endocytosis. In experiments in which receptor recycling was measured, cells were then rinsed with acid wash buffer (Mes-buffered DMEM, pH 5.0; four times with 1 ml each over the course of 10 min) at 4 °C. Hepes-buffered DMEM (1 ml) was then added at 37 °C. In some experiments this buffer was replaced every 5 min with fresh buffer at 37 °C. At the end of the experiment, cells were fixed by incubation for 15 min at room temperature with 0.5 ml of 3% (w/v) paraformaldehyde in PBS. After thorough washing three times with 2 ml of PBS and once with 1 ml of PBS/BSA/HS [PBS containing 1% (w/v) BSA and 1% (v/v) horse serum], cells were incubated with 0.25 ml of anti-HA (approx. 4 μg/ml) in PBS/BSA/HS for 1 h at room temperature, washed three times with 1 ml of PBS/BSA/HS and three times with 2 ml of PBS, incubated with 0.25 ml of alkaline phosphatase-conjugated anti(mouse IgG) (1:150; Vector Laboratories, Peterborough, Cambs., U.K.), washed three times with 1 ml of PBS/BSA/HS

and three times with 2 ml of Tris-buffered saline, then incubated with *p*-nitrophenyl phosphate substrate (Vector Labs) in 100 mM sodium bicarbonate, pH 10, for 10–20 min. Absorbance was measured at 405 nm; the absorbance of the substrate alone was subtracted. A calibration curve was constructed for each experiment where the absorbance was measured from 4000, 5000, 6000, 8000 and 10000 cells per well. Absorbance showed a linear relationship with increasing cell number. At more than 20000 cells per well the antibody concentrations became limiting.

Adenylate cyclase assay

Cells were grown to confluence in 225 cm^2 flasks. Cell monolayers were rinsed twice with PBS, detached from the flask with PBS/EDTA (5 mM) and centrifuged at 500 ***g*** for 5 min. The cells were resuspended in DMEM and distributed into Eppendorf tubes. Each tube contained 0.27 ml of cell suspension (approx. 5×10^5 cells per tube). Cell density was determined by counting the cells with a Coulter counter. Cells were incubated for 15 min with 0.5 mM 3-isobutyl-1-methylxanthine. Forskolin (10 μM final concentration) was added, with drugs where appropriate, for 10 min at 37 °C in a final volume of 0.3 ml. The incubation was terminated by the addition of 10 μl of HCl (10 M) followed by 10 μl of NaOH (10 M) and 200 μl of 1 M Tris/HCl, pH 7.4. The broken cells were pelleted by centrifugation at 12000 ***g*** for 2 min in an Eppendorf centrifuge. The supernatant containing released cAMP was removed and stored at −20 °C.

cAMP was quantified through inhibition of the binding of 2 nM [^{3}H]cAMP to protein kinase A (Sigma; 5 μg per tube) and comparison with a standard calibration curve of known cAMP concentrations from 0.5 to 16 pmol cAMP in a total volume of 0.2 ml. The assay buffer contained 50 mM Tris/HCl, pH 7.4, 4 mM EDTA, 100 mM NaCl and 0.5% BSA. The reaction mixture was incubated for 2 h at 4 °C and the incubation was terminated by filtration through polyethyleneimine-soaked GF/B filters in a Packard 96-tube Filtermate followed by three rinses with 1 ml of ice-cold distilled water. The filters were dried and counted in 50 μl of Microscint O with a Canberra-Packard TopCount scintillation counter.

Data analysis

Concentration–effect curves were fitted to a standard three-parameter logistic equation by using the solver function of Microsoft Excel. Ligand recycling data was fitted to the exponential equation:

$$r = m_r(1 - e^{(-kt)})$$

where r is the percentage of ligand recycled, m_r is the maximum extent of recycling and k is the rate constant for recycling.

Materials

^{125}I-labelled BIM-23027 (specific radioactivity 2000 Ci/mmol) and ^{125}I-Tyr^{11}-SRIF-14 (specific radioactivity 2000 Ci/mmol) were obtained from Amersham International (Little Chalfont, Bucks., U.K.). Cell culture medium was from ICN Biomedicals (High Wycombe, Bucks., U.K.) and fetal bovine serum was from Gibco (Uxbridge, Middx., U.K.). BIM-23027 was synthesized by Dr. J. Kitchen's team (Chemistry Division, Glaxo Group Research, Stevenage, Herts, U.K.). All other chemicals were from Sigma (Poole, Dorset, U.K.). Sep-Pak Plus C_{18} minicolumns were obtained from Waters Chromatography (Watford, Herts., U.K.). Anti-HA antibody was obtained from BABCO via Cambridge Bioscience. All other antibodies and *p*-nitrophenyl phosphate substrate were from Vector Laboratories.

RESULTS

Quantitative analysis of the internalization of the somatostatin agonists ^{125}I-labelled BIM-23027 and ^{125}I-labelled SRIF-14 was achieved through their resistance to a low-pH wash on internalization. Internalization of ligand by non-specific fluid-phase uptake and non-specific binding was determined via the amount of ligand accumulated in the presence of an excess (1 μM) of unlabelled SRIF-14. Conditions were chosen such that the amount of ligand internalized was less than 10% of the total ligand added, so that the rate of internalization would not decrease owing to depletion of ligand. The amount of radioligand internalized increased with time from 5 to 60 min (Figure 1). Internalization of ^{125}I-labelled BIM-23027 was substantially decreased at temperatures lower than 37 °C (Table 1). Both the

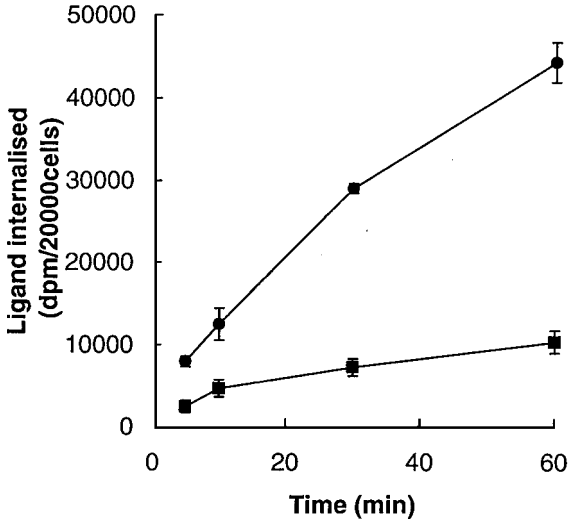

Figure 1 Internalization of ^{125}I-labelled SRIF-14 and ^{125}I-labelled BIM23027

^{125}I-labelled SRIF-14 (■) or ^{125}I-labelled BIM23027 (●) was incubated with cells (2×10^4 cells per well) at 37 °C for various times as shown. Washing with acid was used to remove unbound and surface-bound ligand. Excess unlabelled SRIF or BIM-23027 were used in some wells to determine the amount of non-specific internalization. Results are expressed as radioactivity internalized (d.p.m.) and are means ± S.E.M. for three separate determinations.

Table 1 Internalization and recycling of ^{125}I-labelled BIM-23027 are temperature- and energy-dependent

^{125}I-labelled BIM-23027 was internalized for 5 min at 37 °C for control cells or at the temperature shown. Surface-bound agonist was removed by washing with acid (see the text). For recycling experiments, after internalization for 5 min, cells were washed with acid, then warmed in medium at the temperature shown in the presence of 1 μM SRIF-14 to prevent re-endocytosis. Energy depletion was achieved by pretreatment with antimycin (50 nM) and deoxyglucose (50 mM) for 30 min. The control internalization is the amount of radioligand accumulated inside the cell at 37 °C. The control recycling is the amount of radioligand accumulated extracellularly in 30 min at 37 °C in the presence of 1 μM unlabelled SRIF-14. Results are means ± S.E.M. for *n* observations. Abbreviation: n.d., not determined.

Treatment	Internalization (% of control)	*n*	Recycling (% of control)	*n*
4 °C	3.2 ± 1.2	5	20.9 ± 3.2	3
15 °C	11.2 ± 3.6	5	32.6 ± 10.4	3
Antimycin	13.4 ± 2.3	3	52.3 ± 8.9	3
Sucrose (0.5 M)	19.0 ± 4.5	3	n.d.	3

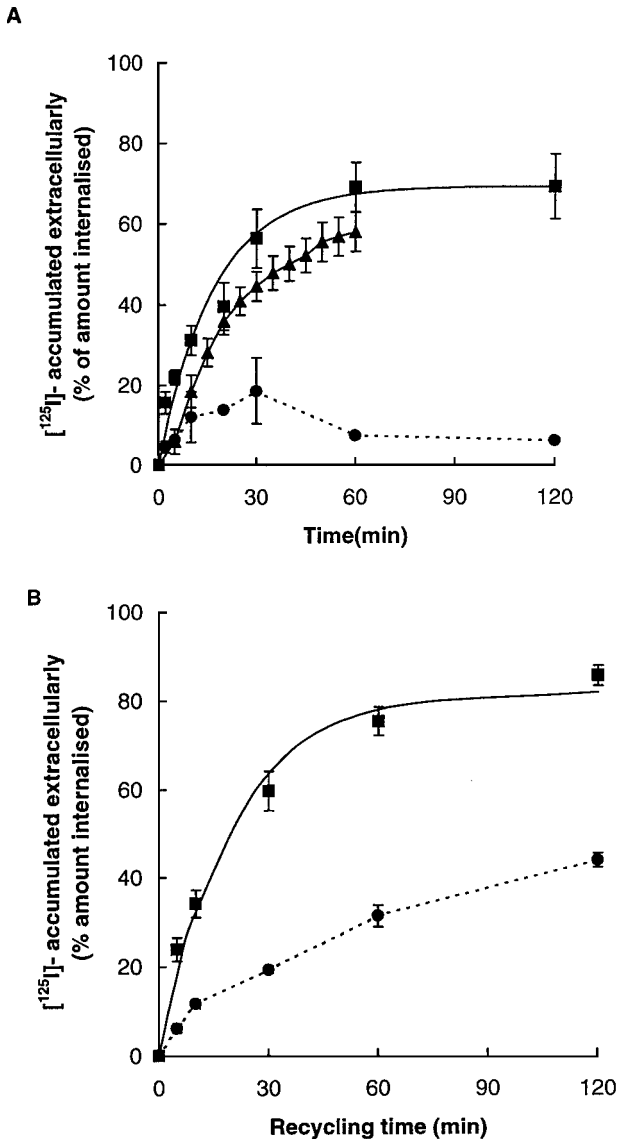

Figure 2 The extent of recycling of internalized ^{125}I-labelled BIM-23027 (A) or ^{125}I-labelled SRIF-14 (B) is limited by re-endocytosis

^{125}I-labelled BIM-23027 or ^{125}I-labelled SRIF-14 was internalized by incubation with cells at 37 °C for 60 min. Washing with acid (pH 5) was used to remove unbound and surface-bound ligand. Excess unlabelled SRIF was included in some wells to determine the amount of non-specific internalization and recycling. Cells were then warmed in 37 °C Hepes-buffered DMEM (1 ml) for the durations indicated in the absence (●) or presence (■) of 1 μM unlabelled SRIF-14 (**A**) or BIM 23027 (**B**) and the external medium containing recycled ligand was collected and quantified in a γ-counter. Alternatively the external medium was removed and collected and replaced with fresh 37 °C medium every 5 min (▲). The amount of radioactivity in each sample was determined and summed cumulatively. Results are expressed as a percentage of the amount of ligand internalized in 60 min and are means ± S.E.M. for three separate determinations. The solid line is fitted to an exponential equation (see the Materials and methods section) with the following kinetic parameters: rate constant 0.06 min^{-1}, maximum 69% (**A**); rate constant 0.05 min^{-1}, maximum 83% (**B**).

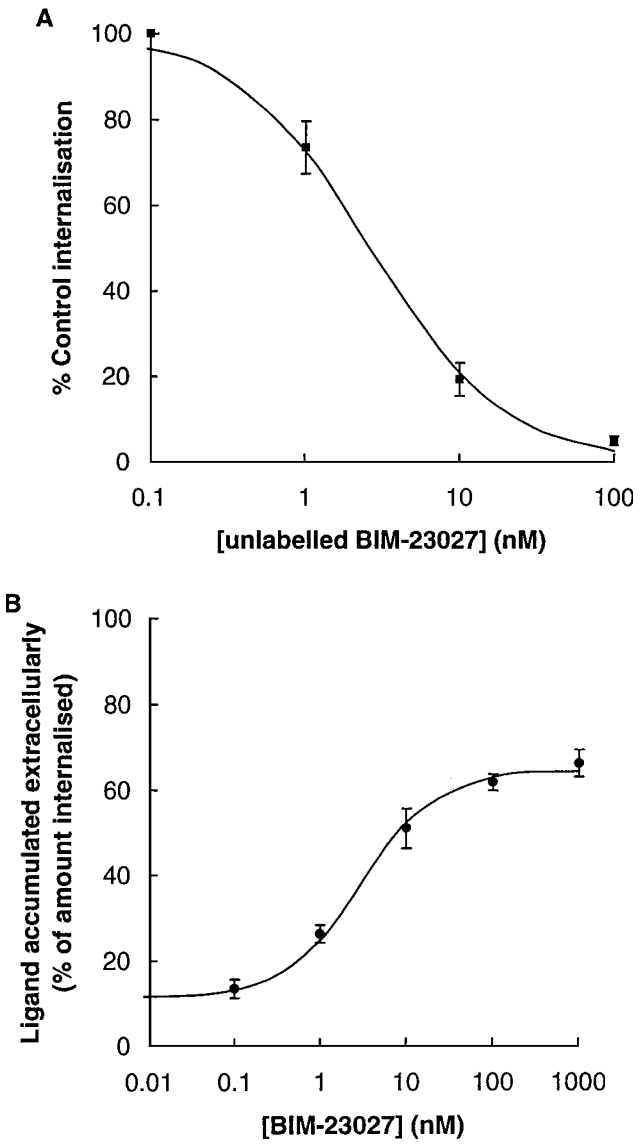

Figure 3 Internalization and re-internalization of ^{125}I-labelled BIM-23027 are prevented by the inclusion of increasing concentrations of unlabelled BIM-23027

For internalization experiments (**A**), cells were incubated with 0.1 nM ^{125}I-labelled BIM-23027 in 0.25 ml of Hepes-buffered DMEM for 5 min at 37 °C with the inclusion of the indicated concentrations of unlabelled BIM-23027. Unbound and surface-bound ligands were removed by washing with 4 °C Mes-buffered DMEM at pH 5 over the course of 10 min. Internalized ligand was solubilized in 1% (v/v) Triton X-100 and transferred to vials for γ-counting. For recycling experiments (**B**), radioligand was internalized as in (**A**) except that the incubation was for 60 min. After being washed with acid, cells were warmed in 37 °C Hepes-buffered DMEM (1 ml) for 60 min in the presence of various concentrations of BIM-23027; the external medium containing recycled ligand was collected and quantified in a γ-counter. In (**A**) results are expressed as a percentage of the amount of ligand internalized in 5 min and are means ± S.E.M. for three separate determinations. The results were fitted to a standard three-parameter logistic with a minimum of 0%, a maximum of 100% and an EC_{50} of 2.7 nM. In (**B**) results are expressed as a percentage of the amount of ligand internalized in 60 min and are means ± S.E.M. for three separate determinations. The results were fitted to a standard three-parameter logistic with a minimum of 11%, a maximum of 64% and an EC_{50} of 2.9 nM. Excess unlabelled SRIF was included in some wells to determine the amount of non-specific internalization and recycling; these values were subtracted to obtain specific internalization and recycling.

depletion of intracellular ATP with antimycin and deoxyglucose and the inhibition of clathrin-coated pit formation by hyperosmolar sucrose significantly inhibited internalization, indicating that somatostatin agonist endocytosis occurs via energy- and clathrin-dependent mechanisms similar to those required for other ligands such as low-density lipoprotein and transferrin (Table 1). Internalization of ^{125}I-labelled SRIF-14 showed similar characteristics (results not shown).

Because other ligands, such as transferrin and EGF, can be recycled back to the extracellular medium, we investigated

Table 2 Estimation of the amount of ^{125}I-labelled SRIF-14 peptide degradation by the proportion of radioactivity eluted with [^{3}H]tyrosine standards

'External medium' is the extracellular medium collected; 'internal medium' is that obtained after removal of extracellular medium and solubilization of cells in 0.1 % (v/v) Triton X-100. The percentage degraded is the proportion of radioactivity in each sample that was eluted with [^{3}H]tyrosine standards. The percentage degradation in samples that were not incubated with cells was 4.6 ± 0.1 %. Note that 111 000 d.p.m. corresponds to 0.25 ml of 0.1 nM ^{125}I-labelled SRIF-14. Results with the same footnote symbols were significantly different from each other ($P < 0.05$).

Internalization time (min)	Recycling time (min)	External medium total/degraded (d.p.m. per 2×10^4 cells)	External medium degraded (%)	Internal medium total/degraded (d.p.m. per 2×10^4 cells)	Internal medium degraded (%)
5	0	108 500/8350	7.7 ± 0.4	$2480 \pm 740/210 \pm 120$	8.5 ± 4.0
5 (+0.5 M sucrose)	0	110 530/7630	6.9 ± 0.9	$470 \pm 140/63 \pm 30$	13.4 ± 4.9
60	0	100 600/32 700	*32.5 ± 3.0	$10\,400 \pm 1340/780 \pm 320$	7.5 ± 2.9
60 (+0.5 M sucrose)	0	109 020/16 240	*14.9 ± 1.7	$1980 \pm 250/260 \pm 130$	13.1 ± 6.2
60	120	$4580 \pm 610/2940 \pm 430$	#64.2 ± 3.9	$5820 \pm 1210/520 \pm 170$	8.9 ± 2.3
60	120 (+excess BIM23027)	$8530 \pm 1100/3080 \pm 510$	#36.1 ± 3.7	$1870 \pm 260/220 \pm 80$	12.0 ± 3.8

whether this was also true of somatostatin agonists. After internalization of ^{125}I-labelled BIM-23027 for 60 min and washing with acid to remove unbound and surface-bound ligand, cells were warmed in medium and the external medium was collected after various durations. The ligand remaining internally was then solubilized and quantified. In all cases the amount of ligand collected externally and the amount of ligand remaining internally added up to between 90 % and 110 % of the amount of ligand internalized after the 60 min internalization incubation. If medium alone was included in the recycling incubation, 10–20 % of the ligand could be collected in the external medium, with 80–90 % remaining inside the cell (Figure 2A). If, however, the external medium was withdrawn and replaced every 5 min with fresh medium, 60 % of the radioactivity could be collected externally, leaving 40 % inside the cell after 60 min. This observation suggested that radioligand was being recycled and re-endocytosed. Inclusion of excess unlabelled SRIF-14 or BIM23027 in the external recycling medium prevented re-endocytosis and allowed 69 ± 8 % of the ligand to be collected externally. A similar experiment was performed with ^{125}I-labelled SRIF-14 (Figure 2B). If re-endocytosis was prevented by the inclusion of excess BIM-23027, 82 ± 1 % of the radioactivity was collected extracellularly after 2 h. The extent of recycling did not depend on the nature of the agonist because in the presence of excess SRIF-14, 83 ± 3 % of radioactivity collected extracellularly at 2 h. However, if medium alone was included in the recycling incubation, 40–50 % of the radioactivity could be collected in the external medium (Figure 2B). The rate constants for recycling (k_r) were very similar for the two agonists, 0.07 ± 0.01 min^{-1} for ^{125}I-labelled BIM-23027 and 0.05 ± 0.01 min^{-1} for ^{125}I-labelled SRIF-14.

To confirm that the apparent inability of ^{125}I-labelled BIM-23027 to be recycled was due to re-endocytosis, the concentration dependence of the accumulation of radiolabel in the medium was determined. The inclusion of various concentrations of unlabelled BIM-23027 showed that the internalization of 0.1 nM ^{125}I-labelled BIM-23027 was prevented in a concentration-dependent manner with an IC_{50} of 2.7 nM (Figure 3A). Figure 3(B) shows that the proportion of ligand recycled was increased by the inclusion of various concentrations of unlabelled BIM-23027 with an EC_{50} of 2.9 nM. The maximum amount of ligand accumulated in the extracellular medium in the presence of 1 μM unlabelled BIM-23027 was 66 ± 3 %. Recycling of both ^{125}I-labelled BIM-23027 and ^{125}I-labelled SRIF-14 was substantially decreased at lower temperatures and by depletion of ATP, conditions that have been shown to decrease the intracellular trafficking of many membrane proteins (Table 1).

Because it has been reported that peptide ligands are largely degraded [31], the fate of ^{125}I-labelled SRIF-14 after endocytosis was compared with the more stable analogue ^{125}I-labelled BIM-23027. Samples of external medium and internal, solubilized radioactivity were subjected to reverse-phase column chromatography, which separates intact peptide from the major degradation product ^{125}I-labelled tyrosine [30]. Samples were taken after either 5 or 60 min of internalization, or after 60 min of internalization then 120 min of recycling. In all samples less than 6 % of ^{125}I-labelled BIM-23027 was eluted with [^{3}H]tyrosine standards. Equivalent results with ^{125}I-labelled SRIF-14 indicated less than 10 % degradation after 5 min of internalization but a significant amount of degradation after 60 min of internalization (32.5 ± 3.0 %; Table 2). Degradation was dependent on the radioligand's being internalized because it could be decreased (from 32.5 % to 14.9 %) by preventing internalization with hypertonic sucrose solution. The proportion of ligand degraded was much higher in the samples of external medium than in internal medium (i.e. that extracted by solubilization of intact cells), suggesting that the degraded form of radioactivity (^{125}I-labelled tyrosine) was extruded from the cell and not re-endocytosed. Analysis of the external medium collected after 2 h of recycling showed that if re-endocytosis was prevented by the inclusion of excess unlabelled BIM-23027, 36 % of the external radioactivity was ^{125}I-labelled tyrosine. In contrast, if re-endocytosis of ^{125}I-labelled SRIF-14 was not prevented, a greater proportion (64 %) of the external radioactivity was accounted for by ^{125}I-labelled tyrosine.

Internalization and recycling studies of radiolabelled agonists generally employ low concentrations of agonist (0.1 nM in this study). Although endocytosis of radioligand occurred at 0.1 nM, higher concentrations (above 1 nM) were required to decrease surface receptor number significantly (Figure 4A). The ability of surface receptor number to recover after agonist-induced endocytosis was dependent on whether or not the extracellular medium was replenished frequently to remove recycled agonist, suggesting that the recycled agonist can initiate the re-endocytosis of receptors (Figure 4B).

Desensitization protocols generally involve the pretreatment of cells with higher concentrations of agonist than that used to measure agonist ligand endocytosis, typically 10 nM to 1 μM. To determine the extent of recycling of agonists after pretreatment of cells with higher concentrations of agonist, the concentration of recycled ligand in the extracellular medium was determined by bioassay. Cells in 24-well plates were treated with 1, 10 or 100 nM BIM-23027 for 60 min at 37 °C. After a thorough washing with acid to remove unbound and surface-bound ligand,

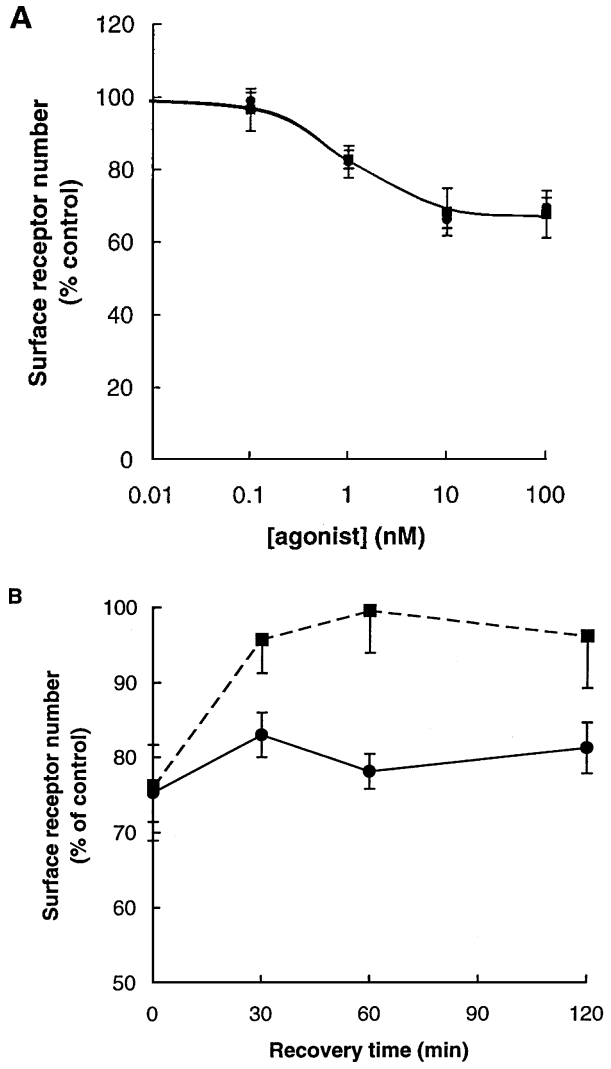

Figure 4 Agonists induce a concentration-dependent loss of surface receptor number that can recover back to control values after washout of agonist provided that recycled agonist is removed

(**A**) Cells were incubated with either BIM-23027 (●) or SRIF-14 (■) for 60 min at 37 °C at the concentrations indicated. (**B**) Cells were incubated with 100 nM BIM-23027 for 60 min at 37 °C to cause internalization. After being washed with acid to remove unbound and surface-bound ligand, cells were warmed in either 1 ml of Hepes-buffered DMEM (●) for the durations indicated or warmed in Hepes-buffered DMEM that was replaced every 5 min (■). After fixation with 3% (v/v) paraformaldehyde, the number of surface receptors was determined by enzyme-linked immunoassay (see the text). Results are expressed as a percentage of surface receptor number in control (untreated) cells and are means ± S.E.M. for five separate determinations. In (**A**) the results were fitted to a standard three-parameter logistic with a minimum of 67%, a maximum of 100% and an EC_{50} of 0.89 nM.

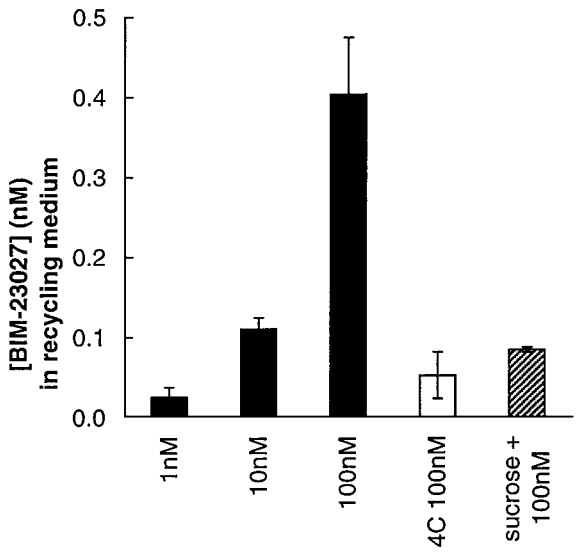

Figure 5 Estimation of the amount of BIM-23027 recycled from the cells after endocytosis by measurement of the inhibition of forskolin-stimulated adenylate cyclase

Cells were incubated with 1, 10 or 100 nM BIM-23027 for 60 min at 37 °C or with 100 nM BIM-23027 at 37 °C in the presence of 0.5 M sucrose or at 4 °C (4C). After being washed with acid to remove unbound and surface-bound ligand, cells were warmed in 0.5 ml of Hepes-buffered DMEM for 10 min. The external medium was collected and added to fresh cells in the presence of forskolin as described in the Materials and methods section. The amount of inhibition of forskolin-stimulated adenylate cyclase was compared with the inhibition due to a known concentration of BIM-23027 to derive the concentration of BIM-23027 in the recycling medium. Results are means ± S.E.M. for three separate determinations.

cells were warmed in Hepes-buffered DMEM for 10 min and the medium was collected. The actual concentration of BIM-23027 in the external medium was calculated from the amount of inhibition of forskolin-stimulated cAMP and compared with the inhibition caused by a known concentration of BIM-23027 in control experiments. In control experiments, the IC_{50} for BIM-23027 was 0.018 nM and the maximum inhibition was 90%. After incubation with agonist (100 nM) for 60 min at 37 °C (followed by washing with acid to remove surface-bound agonist), recycling of agonist occurred such that the concentration in the extracellular medium after 10 min was approx. 0.4 nM (Figure 5). To check that the BIM-23027 in the recycling medium came from previously internalized ligand rather than ligand that had not been removed from the cell surface by washing with acid, the internalization incubation was performed at either 4 °C or 37 °C in the presence of hyperosmolar sucrose, conditions shown to inhibit internalization (Table 1). The concentration of BIM-23027 in the recycling medium after internalization at 4 °C was 9% of that after internalization at 37 °C (Figure 5). The concentration of BIM-23027 in the recycling medium after internalization at 37 °C in the presence of hyperosmolar sucrose was 16% of that after internalization at 37 °C without sucrose (Figure 5). These results demonstrate that the concentration of accumulated recycled agonist was sufficient to continue to activate receptors, leading to the inhibition of forskolin-stimulated adenylate cyclase and re-endocytosis of receptors.

DISCUSSION

The fate of endocytosed agonists and receptors is an important determinant in the regulation of receptor number. Growth factor receptors that show efficient recycling rather than routing to lysosomes for degradation demonstrate slower down-regulation [9]. The sorting of growth factor receptors in endosomes seems to be determined by the affinity of the agonist–receptor interaction at the low pH found in endosomes, with low-affinity agonists causing predominantly receptor and agonist recycling, whereas agonists that remain bound to the receptor are routed to lysosomes for degradation [9]. The situation with G-protein-coupled receptors is made more complex by the presence of two

distinct affinity states of the receptor, high affinity when complexed with G-protein and low affinity without. The combination of this observation with the strong pH dependence of somatostatin agonist binding leads to the suggestion that somatostatin agonists and sst_2 receptors should be predominantly recycled. However, an examination of the literature revealed that although other G-protein-coupled receptors for peptide hormones are generally recycled, the evidence that existed suggested that the ligand was degraded. We wanted to test whether this was true of somatostatin receptors.

Accumulation of radiolabelled somatostatin agonists in the extracellular medium after receptor-mediated endocytosis was small under conditions where re-endocytosis of recycled ligand was not prevented. Re-endocytosis was prevented by either continually removing and replacing the external medium or including a high concentration of unlabelled agonist in the recycling medium, which would be expected to compete with recycled agonist for binding to the receptor. Further evidence that the radioligand was being re-endocytosed came from an experiment in which different concentrations of unlabelled ligand were included in the recycling medium. The EC_{50} value for increasing the amount of ligand collected in the recycling medium was very similar to the IC_{50} for inhibition of endocytosis of radioligand (approx. 3 nM; Figure 3). These results demonstrate that agonist ligands can cycle continuously between the cell surface and intracellular compartments. Furthermore these observations confirm our prediction that because somatostatin agonists dissociate rapidly under conditions likely to be found in endosomes (i.e. at low pH conditions in the presence of cellular concentrations of GTP [25]), the fate of the endocytosed ligand is predominantly recycling rather than degradation. This work underlines the importance of careful experimental design because recycled agonist must be removed to prevent the reactivation of receptors at the cell surface. We have shown that this is particularly important in the measurement of the recovery of surface receptor number after internalization of receptors and in the measurement of desensitization. Furthermore, because receptors are predominantly recycled, down-regulation of total receptor number does not occur within the first 1 h of agonist treatment [indicated by the complete recovery of surface receptor number after washout of agonist and of recycled agonist in Figure 4(B)].

SRIF-14 is the endogenous ligand for the sst_2 receptor and has been reported to be rapidly metabolized by native tissues or cell lines [25,31]. However, it is not clear whether the metabolism occurs inside cells or at the cell surface, an issue not commonly addressed by many authors. We were therefore interested to determine whether SRIF-14 underwent a different fate from that of the more metabolically stable analogue BIM-23027. Although the rate constants for the recycling of both agonists were very similar, ^{125}I-labelled SRIF-14 was degraded to a greater extent than ^{125}I-labelled BIM-23027. The degradation of ^{125}I-labelled BIM23027 was less than 10 % under all conditions but the extent of degradation of ^{125}I-labelled SRIF-14 depended on endocytosis of the ligand because it was decreased by treatment with hyperosmolar sucrose, which prevents clathrin-mediated endocytosis. The most striking difference between ^{125}I-labelled BIM23027 and ^{125}I-labelled SRIF-14 was observed if re-endocytosis of agonist was not prevented. In this case the accumulation of extracellular radioactivity was much greater for ^{125}I-labelled SRIF-14 (44 % after 2 h) than for ^{125}I-labelled BIM-23027 (6 % after 2 h). This difference can be explained by the greater proportion of the major degradation product ^{125}I-labelled tyrosine in the experiments with ^{125}I-labelled SRIF-14. Tyrosine is not internalized efficiently in these cells (J. A. Koenig, unpublished work), so the re-endocytosis of ^{125}I-labelled SRIF-14 would lead to a decrease in the total amount of radioactivity outside the cell and a decrease in the proportion of radioactivity accounted for by intact rather than degraded agonist. This finding has important consequences for the behaviour of radioisotope-conjugated agonists in the treatment of tumours overexpressing somatostatin receptors because the use of the more stable analogue is likely to lead to a greater accumulation of radioactivity inside the cell.

We propose a model, based on our findings, in which somatostatin receptor agonists are endocytosed and accumulate in sorting endosomes. From these structures, most of the ligand is recycled back to the cell surface, whereas a small proportion is degraded while inside the cell. Exactly where degradation takes place is as yet unknown. The recycled agonist can then be re-endocytosed and continue cycling to and from the cell surface unless steps are taken to prevent re-endocytosis. This finding that most of the internalized agonist is recycled rather than degraded in lysosomes is in contrast with the conclusions from studies with fluorescently labelled agonist ligands for the angiotensin receptor [17] and the gastrin/CCK-B receptor [22], for which fluorescence was demonstrated in lysosomes. Preliminary studies with fluorescein–SRIF suggest that somatostatin agonists behave similarly because fluorescence remains inside the cell for up to 2 h after washout. In our experiments with ^{125}I-labelled agonist, a proportion of the radioactivity remained inside the cell after washout, depending on the extent of degradation of the agonist and the experimental conditions. It is possible that the ligand remaining inside the cell could be enough to account for the observed fluorescence in the studies of the angiotensin receptor [17] and the gastrin/CCK-B receptor [22]. Alternatively, in these studies, the majority of the ligand might have recycled to the surface and re-endocytosed or diffused away and become undetectable.

Internalization studies with radio-iodinated agonists are commonly performed with relatively low concentrations of ligand (0.05–0.2 nM). It is possible that at such low concentrations a higher proportion of ligand could be recycled than at higher concentrations. We wanted to know whether, at the concentrations of agonist commonly employed to induce receptor internalization and desensitization (1–100 nM), agonists were also recycled to the same extent. We found that the concentration achieved (approx. 0.4 nM) was more than enough to affect the recovery of surface receptor number. However, 0.4 nM BIM-23027, if distributed evenly through the medium, would not be enough to stimulate receptor internalization maximally. Therefore we can conclude that either ligand molecules are recycled with receptors or that the recycled ligand remains in an unstirred layer near the cell surface, creating an apparently higher concentration that would be sufficient to initiate maximal internalization.

These results have important implications for the design of experiments involving the pretreatment of cells with high concentrations of somatostatin followed by measurement of surface receptor number by ligand binding (internalization studies) or functional responses (desensitization studies). Furthermore the recycling of internalized ligand after washout of surface-bound ligand is likely to inhibit subsequent radioligand binding assays, making the results of such experiments unreliable. The recycling step showed less sensitivity to lower temperature and ATP depletion than internalization, which implies that a reliance on lower temperature to prevent recycling during the quantification of surface receptor number by ligand binding is likely to generate artifacts. It was for this reason that an enzyme-linked immunoassay was employed in this study to measure surface receptor

number. Measurement of functional responses after pretreatment with 100 nM BIM-23027 or SRIF-14 is also likely to be affected by the accumulation of recycled ligand. Indeed, the forskolin-stimulated accumulation of cAMP in agonist-pretreated cells is less than in untreated cells (results not shown). These findings show that the analysis of desensitization studies is difficult if recycling and re-endocytosis of ligands and receptors are not taken into account.

In conclusion, we have shown that somatostatin agonists and the sst_2 somatostatin receptor are predominantly recycled after receptor-mediated endocytosis in CHO cells rather than routed to lysosomes for degradation. This observation is important for two reasons. First, it is important in the design of experiments with desensitization protocols because the recycled ligand might be of sufficient concentration to reactivate receptors at the cell surface and regulate their number and distribution. Second, it is important to understand the endocytosis and recycling kinetics of agonist ligands to understand how the concentration of ligand both inside and outside the cell is controlled. It has been proposed that receptor-mediated endocytosis of peptide agonists might be responsible for decreasing the concentration of ligand in the extracellular space; similar arguments for 'clearance' receptors have been proposed [33]. However, this is unlikely to be true of somatostatin receptors because we have shown that somatostatin agonists are predominantly recycled. Furthermore the accumulation of radioisotope-conjugated somatostatin analogues is used in the treatment of certain tumours, particularly in the gastrointestinal tract, and detailed knowledge of the kinetics of both receptor and ligand endocytosis and recycling are necessary for the design of effective treatments [34]. Indeed, one can speculate that the essential characteristic of an agonist ligand that will accumulate most effectively inside cells will be that it does not dissociate readily in the low pH of endosomes.

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