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Somatostatin receptors in Neuro2A neuroblastoma cells: ligand internalization

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1 Receptor-dependent internalization of somatostatin (SRIF) agonists has been a matter of controversy probably because [^{125}I]-Tyr^{11}-SRIF-14 is rapidly degraded. We have studied the internalization of a stable somatostatin analogue, [^{125}I]-BIM-23027, in a neuronal cell line, Neuro2A, which natively expresses somatostatin sst_2 receptors.

2 Incubation of Neuro2A cells with [^{125}I]-BIM-23027 at 37°C resulted in a time-dependent internalization of the ligand, which reached a maximum at 30 min. Acid-washing showed that cell-surface binding of the ligand accounted for only 34% of total binding at this time. internalization was dramatically reduced at 15°C.

3 internalization of [^{125}]-BIM-23027 was prevented by inclusion of unlabelled somatostatin receptor agonists in a concentration-dependent manner. The IC_{50} values for inhibition of [^{125}I]-BIM-23027 internalization were approximately 100 fold lower than for inhibition of [^{125}I]-BIM-23027 binding to membrane homogenates but followed the same rank order of potencies.

4 Disruption of G-protein coupling by treatment with pertussis toxin caused a 60% reduction in internalization of ligand. A combination of antimycin (50 nM) and deoxyglucose (50 mM) pretreatment, which leads to a depletion of cellular ATP, decreased internalization of [^{125}I]-BIM-23027 by 66% of control and increased the proportion of surface-bound ligand. Hypertonic sucrose, which prevents clathrin-mediated endocytosis, reversibly abolished the internalization of ligand without increasing the proportion bound at the cell surface.

5 After internalization of [^{125}I]-BIM-23027, approximately half of the ligand was recycled back to the extracellular medium within 20 min at 37°C. This finding suggests that the intracellular content of [^{125}I]-BIM-23027 reaches a steady state which is determined by the rates of both internalization and recycling of the ligand. In contrast to studies in which the internalization of [^{125}I]-Tyr^{11}-SRIF-14 was examined, neither internalized nor recycled [^{125}I]-BIM-23027 was degraded to its component amino acids.

6 These findings indicate that the somatostatin agonist, [^{125}I]-BIM-23027, is internalized in a receptor-dependent manner which involves clathrin-coated pits in Neuro2A cells. Furthermore, much of the internalized ligand is rapidly recycled back to the extracellular medium without undergoing significant degradation.

Keywords: [^{125}I]-BIM-23027; somatostatin receptors; Neuro2A cells; neuroblastoma; internalization; endocytosis

Introduction

The binding of a number of peptide ligands to their endogenous G-protein-coupled receptors has been shown to be followed by internalization of peptide both in neuronal cells (e.g. substance P, Mantyh *et al*., 1995; neurotensin, Beaudet *et al*., 1994) and in host cells containing recombinant receptors (e.g. gastrin-releasing peptide receptor, Benya *et al*., 1994; cholecystokinin receptor, Roettger *et al*., 1995). The functional importance of this process has been postulated to involve desensitization and/or resensitization (Hunyady *et al*., 1991; Barak *et al*., 1995; Pippig *et al*., 1995), morphological changes (Mantyh *et al*., 1995), signal compartmentalization (Baass *et al*., 1995), retrograde signalling (Laduron, 1995) and clearance of the peptide from the extracellular medium (Wu-Wong *et al*., 1995). Significant internalization generally occurs within 10 min and, in some instances, can lead to down-regulation of the number of receptors (Benya *et al*., 1994). In some cases, recycling of the receptor has been demonstrated after removal of agonist (Koenig & Edwardson, 1994a,b).

Although internalization has been demonstrated for other neuropeptides, the ability of somatostatin (SRIF) to be internalized has been a matter of controversy. Presky & Schonbrunn (1988) showed that neither [^{125}I]-Tyr^{1}-SRIF nor [^{125}I]-Tyr^{11}-SRIF was rapidly internalized in GH_4C_1 pituitary cells. Similarly, Sullivan & Schonbrunn (1986) showed that [^{125}I]-Tyr^{11}-SRIF was not internalized in RINm5F insulinoma cells. In both cases, there was significant degradation of the peptide. In contrast, internalization of SRIF has been demonstrated in pancreatic acini (Viguerie *et al*., 1987) and internalization of [^{125}I]-Tyr^{3}-octreotide and [^{125}I]-SRIF-28 have been shown in pituitary tumour cells (Morel *et al*., 1986; Hofland *et al*., 1995). In pancreatic acini, there was also some degradation of the ligand (Viguerie *et al*., 1987). Radiotherapy, with somatostatin analogues coupled to α- or β-emitters, has been proposed as a potential therapeutic strategy (Lamberts *et al*., 1991; Hofland *et al*., 1995). The success of this approach must rely upon the amount of radioligand which can be concentrated within the tumour cells, and this will be determined by the rates of internalization, degradation and recycling of both ligand and receptor.

We were interested to determine whether the cyclic hexapeptide, [^{125}I]-BIM-23027, binds to the cell surface and is internalized in a receptor-dependent manner in the neuroblastoma cell line, Neuro2A. We have also examined

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whether the peptide is degraded either at the surface or inside the cell, and whether it is recycled back into the extracellular medium. A preliminary account of this work has been presented to the British Pharmacology Society (Koenig *et al*., 1996b).

Methods

Cell culture

Neuro2A cells were grown and passaged as described in the accompanying manuscript (Koenig *et al*., 1996a). For experiments with intact cells, cells were grown in 24-well plates and used at confluence.

Membrane preparation

Cells were detached from flasks with PBS-EDTA (phosphate buffered saline (mM) NaCl 137, KCl 2.7, Na_2HPO_4 8.1 and KH_2PO_4 1.5, pH 7.2, EDTA (ethylenediaminetetraacetic acid)), centrifuged and resuspended in assay buffer (10 mM HEPES (N-[2-hydroxyethyl]piperazine-N′-[2-ethanesulphonic acid) pH 7.4, 11 mM $MgCl_2$, 1 mM EDTA containing protease inhibitors bacitracin 0.2 mg ml^{-1}, leupeptin 10 μg ml^{-1} and soybean trypsin inhibitor 1 μg ml^{-1}. After homogenization in a Dounce homogenizer, the cell membranes were pelleted by centrifugation at 20,000 *g* for 20 min and the pellet resuspended in assay buffer and stored frozen at $-20^{\circ}C$ (Koenig *et al*., 1996a).

Ligand binding assays

Membranes (5–15 μg protein) were incubated with [^{125}I]-BIM-23027 in assay buffer for varying times and temperatures as indicated. For assays at pH 5.0, the assay buffer also contained 10 mM MES (2-[N-morpholino]ethane sulphonic acid). Non-specific binding was defined by the inclusion of 2 μM SRIF-14. Incubations were terminated by filtration (Koenig *et al*., 1996a). For association experiments, membranes were incubated for varying times at the appropriate temperature. For dissociation experiments, membranes were incubated with 0.05 nM [^{125}I]-BIM-23027 for 30 min at 37°C and the dissociation was initiated by addition of an equal volume of buffer containing 4 μM SRIF-14. In some experiments, the dissociation buffer also contained GTP (guanosine 5′-triphosphate, 10 μM) and sodium chloride (120 mM). Inhibition curves and association and dissociation kinetic data were analysed by non-linear curve fitting (Koenig *et al*., 1996a).

internalization of [^{125}I]-BIM-23027

Neuro2A cells, grown to confluence on 24-well plates, were washed twice with phosphate buffered saline (PBS), containing 0.9 mM $CaCl_2$ and 0.5 mM $MgCl_2$ (PBS-Ca,Mg), and 0.2 ml incubation buffer was added. The incubation buffer contained PBS-Ca,Mg and (except for experiments studying degradation of ligand) protease inhibitors (leupeptin 10 μg ml^{-1}, soybean trypsin inhibitor 1 μg ml^{-1}) and was pre-warmed to 37°C. The cells were incubated at 37°C for 10 min, then 0.05 ml of 1 nM [^{125}I]-BIM-23027, containing 0.2 mg ml^{-1} bacitracin, was added and the incubation was continued for a further 10 min (unless otherwise specified). Non-specific internalization was determined by the inclusion of 2 μM SRIF-14. Specific internalization typically accounted for 50–60% of total ligand internalized. The incubation was terminated by removing the incubation medium and washing rapidly twice with 2 ml cold PBS-Ca,Mg. A further 2 ml acid-wash buffer was added to dissociate surface-bound ligand. The acid-wash buffer (pH 5.0) contained (in mM): HEPES 10 (pH 5.0), MES 10, NaCl 120, $MgCl_2$ 0.5 and $CaCl_2$ 0.9 and was kept on ice. The cells were incubated in this buffer for 10 min (unless otherwise stated), and then washed with a further 2 ml acid-wash buffer. The internalized radioactivity was released by incubation with 0.25 ml 0.5% (v/v) Triton X-100 for at least 2 h, transferred to vials and counted in a gamma counter. Cell density was determined by removing cells from the wells with 0.25 ml PBS containing EDTA (5 mM) and counting the cells with a Neubauer haemocytometer. There were typically $0.8-1.2\times10^6$ cells/well. internalized ligand accounted for less than 10% of total ligand added.

To determine the proportion of ligand bound to the cell surface and the proportion internalized, cells were incubated with 0.2 nM [^{125}I]-BIM-23027 for 30 min at 37°C, washed rapidly twice with 2 ml cold PBS-Ca,Mg and then incubated with 2 ml of either PBS-Ca,Mg at pH 7.4 or acid-wash buffer at pH 5.0 for varying times. The temperature was maintained at 15°C to prevent further internalization and recycling of receptors. The effect of increasing concentrations of unlabelled agonists on internalization of [^{125}I]-BIM-23027 was determined by co-incubation of agonists with 0.2 nM [^{125}I]-BIM-23027 for 10 min then washing with pH 5.0 buffer as described above.

Treatment with inhibitors of internalization

Intact cells in 24-well plates were preincubated with 0.5 M sucrose for 10 min at 37°C. In some experiments to show reversibility of this effect, the sucrose was removed by washing twice with 2 ml PBS-Ca,Mg. Membranes were treated in a similar way, but washed by centrifugation and resuspension in fresh PBS-Ca,Mg. Cells were depleted of adenosine 5′-trisphosphate (ATP) by incubation with 50 nM antimycin and 50 mM 2-deoxyglucose at 37°C for 30 min before the internalization assay (Hertel *et al*., 1986; Hoover & Toews, 1989). Antimycin and 2-deoxyglucose were contained in all subsequent incubation buffers.

Ligand recycling

Cells were incubated with 0.2 nM [^{125}I]-BIM-23027 for 10 min at 37°C, and then washed thoroughly with pH 5.0 buffer to remove surface-bound ligand. Cells were then warmed to 37°C with fresh incubation buffer containing 1 μM SRIF-14. The excess unlabelled ligand was included to prevent reuptake of recycled radiolabelled ligand. After further washing at pH 5.0, the radioactivity remaining was measured.

Separation of intact from degraded peptide ligand

Peptides were separated from amino acid degradation products by a method based on that of Bohlen *et al*. (1980). A Sep-Pak Plus C18 minicolumn was washed with 4 ml solution B (acetonitrile : water : trifluoroacetic acid, 900 : 100 : 0.5, by vol) and then with 4 ml solution A (water : trifluoroacetic acid, 1000 : 1, by vol). The sample was loaded and salts were eluted with 2 ml 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 80% A and 20% B (fraction 3). By use of radiolabelled standards, it was shown that more than 95% of [^{3}H]-tyrosine eluted in the amino acid fractions (fraction 2) and greater than 95% of [^{125}]-BIM-23027 eluted in the intact peptide fraction (fraction 3). A small proportion (10%) of [^{125}I]-SRIF-14 eluted in fraction 1, which might be accounted for by free iodide; the remainder eluted in fraction 3.

Data analysis

Concentration-effect curves for inhibition of ligand internalization and association and dissociation kinetic experiments were analysed by GraphPad Prism. Concentration-effect curve data were fitted to the equation

$$\text{Bound} = \frac{100}{1 + 10^{(\log[D] - \log(IC_{50}))}}$$

where [D] is the unlabelled ligand concentration.
pIC_{50} values quoted are equal to the $-\log IC_{50}$ value.
Association data were fitted to the equation,

$$Bound = \max imum(1 - e^{-k_{on,apparent}t})$$

$$\text{where } k_{on,apparent} = k_{+1}[D] + k_{-1}$$

Dissociation data were fitted to the equations,
one-site model: $Bound = proportion_1 e^{-k_{-1}t} + asymptote$
two-site model: $Bound = proportion_1 e^{-k_{-1}t} + proportion_2 e^{-k_{-2}t} + asymptote$

Where 'proportion' is the percentage of total binding which dissociated at rate 'k' and 'asymptote' is the percentage of binding which could not be dissociated. The model which fitted the data best was determined by F-test.

Materials

[^{125}I]-BIM-23027 (specific activity, 2,000 Ci mmol^{-1}) and [^{125}I]-Tyr^{11}-SRIF-14 (specific activity 2,000 Ci mmol^{-1}) were obtained from Amersham International plc (Amersham, Bucks). Cell culture medium was from ICN Biomedicals (High Wycombe, Bucks), and foetal bovine serum was from GIBCO (Uxbridge, Middx). Pertussis toxin was from Calbiochem. Peptides were synthesized by Dr J. Kitchen's team, Chemistry Division, Glaxo Group Research: BIM-23027 (*cyclo*[N-Me-Ala-Tyr-D-Trp-Lys-Abu-Phe]), BIM-23056 (D-Phe-Phe-Tyr-D-Trp-Lys-Val-Phe-D-Nal-NH_2) MK-678 (*cyclo*(N-Me-Ala-Tyr-D-Trp-Lys-Val-Phe)) and L-362855 (*cyclo*[Aha-Phe-Trp-D-Trp-Lys-Thr-Phe). All other chemicals were from Sigma (Poole, Dorset). Sep-Pak Plus C18 minicolumns were obtained from Waters Chromatography (Watford, Hertfordshire, U.K.).

Results

Radioligand binding in whole cells

At pH 7.4 in intact, adherent cells, 20% of the specifically bound [^{125}I]-BIM-23027 dissociated with a half-time of 5 min, while at pH 5.0, 34% of the specifically bound ligand dissociated with a half-time of 8 min (Figure 1). Washout kinetic data best fitted a single exponential decay with rate constants and asymptotes respectively, pH 7.4, 0.14 ± 0.12 min^{-1}, 79.6 ± 4.2%; pH 5.0, 0.087 ± 0.072 min^{-1}, 65.8 ± 6.4% (mean $\pm$ s.e.mean, $n = 3$). There was no significant difference at the 5% confidence level between the fits to the two sets of data (F-test, $P = 0.05$) indicating that there was no significant difference between the asymptote after washing at pH 7.0 (79.6%) and the asymptote after washing at pH 5.0 (65.8%). The ligand remaining after 10 min at the lower pH was assumed to represent internalized ligand and the ligand that dissociated within 10 min was assumed to be cell surface-associated.

Radioligand binding in cell homogenates

The kinetics of binding of [^{125}I]-BIM-23027 to membrane homogenates at pH 5.0 was examined. Association of [^{125}I]-BIM-23027 was notably temperature-dependent such that significant binding could be demonstrated at 15°C, but very little binding was observed at 37°C (Figure 2a). The apparent association rates were 0.29 ± 0.12 min^{-1} ($t_{1/2} = 2.4$ min) at 37°C and 0.080 ± 0.007 min^{-1} ($t_{1/2} = 8.6$ min) at 15°C. The maximal binding was 1.5 ± 0.2 fmol mg^{-1} protein at 37°C and 16.0 ± 0.5 fmol mg^{-1} protein at 15°C. Dissociation kinetics also showed a marked temperature dependence. At 37°C, all of the ligand (98.4 ± 4.0%) dissociated from the membranes whereas at 15°C, only 68.1 ± 4.5% of the ligand dissociated (Table 1, Figure 2b). In the absence of GTP and NaCl, data were fitted best by a one-phase dissociation model (Table 1). In order to determine the rate at which the ligand dissociated in the presence of GTP (guanosine 5′-triphosphate, 10 μM) and NaCl (120 mM), the membranes were incubated with [^{125}I]-BIM-23027 for 30 min at 37°C, pH 7.4 without GTP and NaCl to allow equilibrium to be reached, and then the amount of bound ligand remaining was measured after addition of 10 μM GTP, 120 mM NaCl and 2 μM SRIF-14 at a final pH of 5.0. When the dissociation was carried out at 4°C, 82.7 ± 3.0% of the ligand dissociated within 10 min at 15°C, 89.8 ± 1.3% and at 37°C, 98.2 ± 1.7% of the ligand dissociated within 10 min (Figure 2c). In the presence of GTP and NaCl the data were fitted best by a one-phase model at 4°C and a two-phase model at 15°C and 37°C. The kinetic data are shown in Table 1. The relatively high level of non-specific binding precluded the determination of the affinity at pH 5.0 and 37°C.

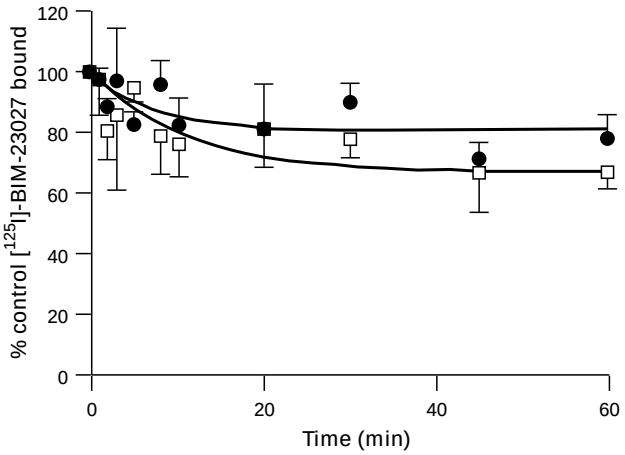

Figure 1 Time course of the removal of surface-bound [^{125}I]-BIM-23027 from Neuro2A cells. Cells were incubated with 0.2 nM [^{125}I]-BIM-23027 at 37°C for 30 min then washed rapidly. Cells were incubated for various times at 15°C with either PBS-Ca,Mg, pH 7.4 (●), or HEPES-MES-buffered saline, pH 5.0 (□). Cells containing internalized ligand were solubilized and transferred to vials for counting in a gamma counter. Data are expressed as the mean percentage of the ligand bound after two rapid (< 10 s) washes with PBS-Ca,Mg. Vertical lines represent s.e.mean ($n = 3$). Lines shown are the best fit to a single exponential decay.

Inhibition of ligand internalization

The internalization of [^{125}I]-BIM-23027 was time-dependent, reaching an asymptote after 30 min at 37°C (Figure 3). internalization was also temperature-dependent, being markedly reduced at 15°C (Figure 3). Internalization of [^{125}I]-BIM-23027 was prevented by the co-incubation of increasing concentrations of a number of somatostatin agonists (see Table 2, Figure 4).

Upon depletion of ATP by pretreatment with antimycin (50 nM) and deoxyglucose (50 mM), there was a marked decrease in the amount of ligand internalized. The amount of radioligand remaining in ATP-depleted cells after a 30 min wash with pH 7.0 buffer was 45 ± 4% (s.e.mean, $n = 3$) of that remaining in control cells. ATP depletion also caused an increase in the amount of ligand that could be dissociated by washing (compare asymptotes in Figures 1 and 5). Fitting the data to a single exponential decay model gave a rate constant 0.41 ± 0.11 min^{-1}, and an asymptote of 33.7 ± 3.7%. Fitting to a more complex model did not provide a better fit.

Pertussis toxin pretreatment (500 ng ml^{-1}) partially inhibited internalization to 40 ± 9% of control. Hyperosmolar sucrose (0.5 M) totally inhibited internalization of [^{125}I]-BIM-23027. Ligand internalization in the presence of 0.5 M sucrose

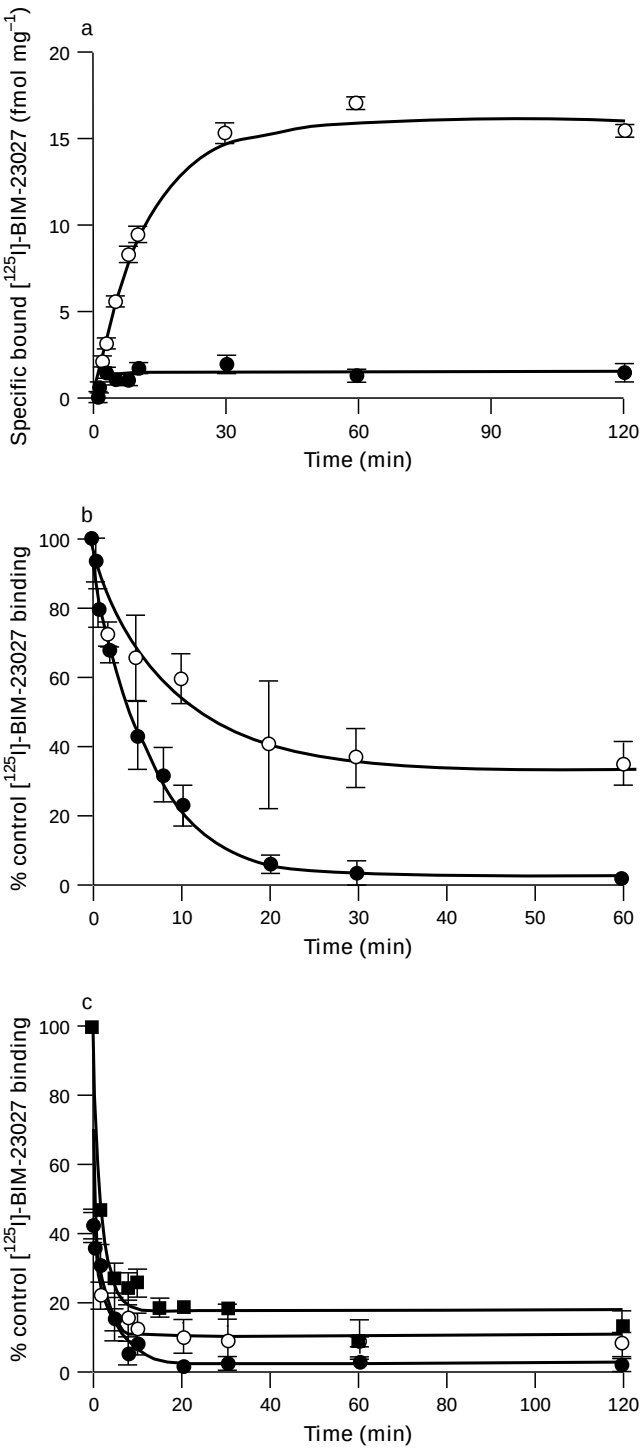

Figure 2 Association and dissociation kinetics of [^{125}I]-BIM-23027 binding to membranes at either 15°C or 37°C at pH 5.0. For association experiments (a), Neuro2A membranes were incubated with [^{125}I]-BIM-23027 (0.05 nM) at either 15°C (○) or 37°C (●). Data are expressed as specific binding (fmol mg^{-1} protein) and represent a typical experiment, which was repeated three times. The points represent the means ± s.e.mean of triplicate determinations. The apparent association rates were 0.29 min^{-1} at 37°C and 0.080 min^{-1} at 15°C. The maximal binding was 1.5 fmol mg^{-1} protein at 37°C and 16.0 fmol mg^{-1} protein at 15°C. (b) and (c) Dissociation kinetics of [^{125}I]-BIM-23027 bound to Neuro2A membranes at pH 5.0 at either 4°C (■), 15°C (○) or 37°C (●). Neuro2A membranes were incubated with 0.05 nM [^{125}I]-BIM-23027 for 30 min at 37°C. In (b) dissociation was initiated with excess SRIF-14. In (c) dissociation buffer also contained 10 μM GTP and 120 mM NaCl. Data are expressed as a percentage of control (no dissociation buffer added and filtered immediately). In the absence of GTP and NaCl, data were fitted best by a one-phase dissociation model. In the presence of GTP and NaCl the data were fitted best by a two-phase model. Kinetic parameters are shown in Table 1. The points represent the means ± s.e.mean of 3 separate determinations. Vertical lines represent s.e.mean.

was reduced to 3 ± 3% (n = 3) of control. This effect was partially reversible, since if the cells were preincubated with 0.5 M sucrose and then washed (2 × 2 ml PBS,Ca,Mg over 5 min), internalization was reduced by only 22 ± 6% of control (n = 3). Inclusion of 0.5 M sucrose in a membrane binding assay resulted in a modest inhibition of specific [^{125}I]-BIM-23027 binding (33 ± 5%; n = 3).

Fate of internalized ligand

Samples of the extracellular medium and internalized ligand were analysed for the presence of amino acid degradation products by use of Sep-Pak Plus C18 minicolumn chromatography which separates [^{125}I]-tyrosine from peptides (Bohlen *et al*., 1980). After incubation of cells with [^{125}I]-BIM-23027 for either 10 or 60 min at 37°C, less than 5% of the radioactivity in the extracellular medium eluted in the [^{125}I]-tyrosine fraction. Furthermore, less than 5% of the internalized radioactivity eluted in the [^{125}I]-tyrosine fraction.

In contrast, [^{125}I]-Tyr^{11}-SRIF-14 was rapidly (< 10 min) degraded after incubation with Neuro2A cells at 37°C. The degradation of [^{125}I]Tyr^{11}-SRIF-14 in the extracellular medium was 69 ± 2% (n = 3) and this could be reduced by the presence of excess unlabelled SRIF-14 (2 μM, 16.6 ± 0.5% degraded, n = 3) or bacitracin (0.16 mg ml^{-1}, 11.6 ± 0.1% degraded, n = 3), but a variety of other protease inhibitors were inactive (greater than 60% degraded, namely leupeptin, phosphoramidon, amastatin, soybean trypsin inhibitor) (Figure 6). The degradation (51 – 59%) of internalized [^{125}I]-Tyr^{11}-SRIF-14 analysed after Triton-extraction from the cells was not affected by any of the protease inhibitors (Figure 6). This inclusion of more than 0.04 mg ml^{-1} bacitracin resulted in a significant loss of cells after an incubation period of 60 min or longer.

Recycling of internalized ligand was assessed by use of a 'pulse-chase' protocol. After incubation of cells with [^{125}I]-BIM-23027 for 10 min, the cells were treated with acid-wash buffer for 10 min, and then incubated at 37°C for various times. Approximately half of the internalized ligand was recycled into the extracellular medium within 20 min (Figure 7). Analysis of the retained and recycled ligand showed that, in both cases, less than 5% of the radioactivity eluted in the [^{125}I]-tyrosine fraction, suggesting that no degradation had occurred.

Discussion

In insulinoma cells and in GH_4C_1 cells [^{125}I]-Tyr^{11}-SRIF is degraded at the cell surface rather than being internalized while in pancreatic acini, [^{125}I]-Tyr^{11}-SRIF is internalized but also rapidly degraded (Sullivan & Schonbrunn, 1986; Viguerie *et al*., 1987; Presky & Schonbrunn, 1988). In contrast, the octapeptide somatostatin analogue, [^{125}I]-octreotide, is internalized in pituitary tumour cells without significant degradation; but the ability of the ligand to be recycled was not assessed (Hofland *et al*., 1995). We have examined a neuroblastoma cell line, Neuro2A, to determine whether or not [^{125}I]-Tyr^{11}-SRIF or [^{125}I]-BIM-23027 are degraded or internalized in a receptor-dependent manner and to investigate the mechanisms involved. The cell line chosen has the advantage that it contains sst_2 receptors which have been characterized (Koenig *et al*., 1996a) and that [^{125}I]-BIM-23027, a cyclic hexapeptide, is available as a novel, stable and selective sst_2 receptor radioligand (Holloway *et al*., 1996).

Validation of the acid wash method

Internalization of a ligand is generally assessed by the removal of surface bound ligand by acid washing. However, given that some peptide ligands do not fully dissociate after binding to membrane preparations, care must be taken to ensure that any ligand that is resistant to acid washing is actually located internally rather than tightly bound to the surface. Choosing the best conditions involves a compromise between washing all of

Table 1 Dissociation rates for [^{125}I]-BIM-23027 binding in Neuro2A membranes

Temp (°C)	$k_{-1,site1}$ (*min*$^{-1}$)	*Proportion site 1* (%)	$k_{-1,site2}$ (*min*$^{-1}$)	*Proportion site 2* (%)	*Asymptote* (%)	n
15	0.115±0.034	64.7±6.7			31.9±4.5	3
37	0.16±0.02	95.7±4.0			1.6±2.9	3
In presence of GTP (10 μM) and sodium chloride (120 mM)						
4	0.47±0.08	81.9±5.6			17.3±2.1	3
15	>5	48.2±10.6	0.50±0.18	41.6±9.8	10.2±1.3	3
37	0.24±0.05	44.4±4.6	8.5±13.7	53.9±5.5	1.8±1.7	3

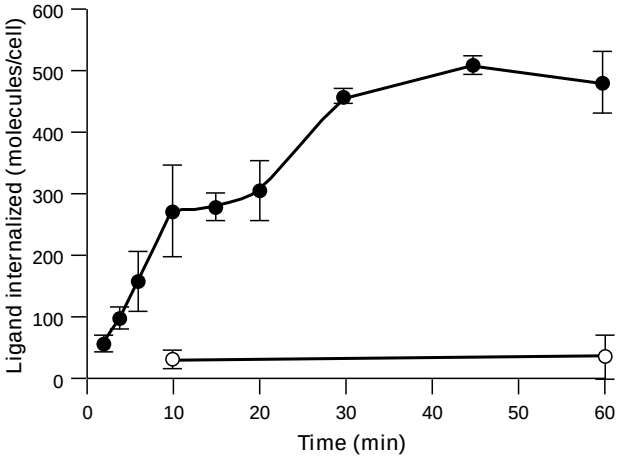

Figure 3 Time dependence of internalization of [^{125}I]-BIM-23027. Cells were incubated with [^{125}I]-BIM-23027 (0.2 nM) at either 37°C (●) or 15°C (○) for various times and washed thoroughly with pH 5.0 buffer to remove surface-boud ligand. internalized ligand was solubilized and transferred to vials for gamma counting. Data are expressed as the number of ligand molecules internalized per cell. The points represent the means±s.e.mean of three separate determinations.

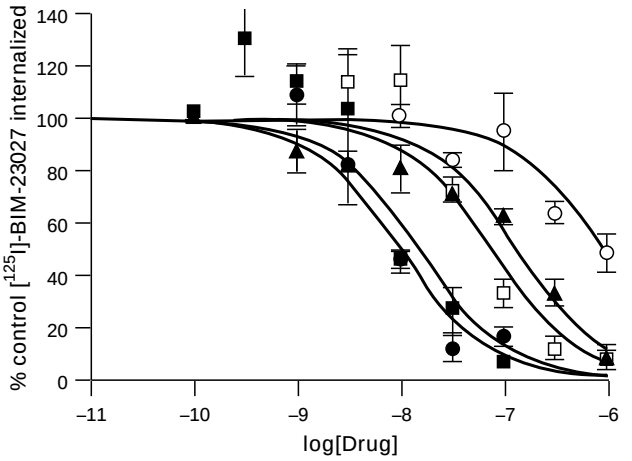

Figure 4 Inhibition of internalization of [^{125}I]-BIM-23027 in Neuro2A cells by incubation with unlabelled analogues. Cells were incubated with 0.2 nM [^{125}I]-BIM-23027 at 37°C with or without unlabelled analogues for 10 min: BIM-23056 (○); BIM-23027 (●); SRIF-14 (■); SRIF-28 (□); L-362855 (▲). After thorough washing to remove surface-bound ligand, cells were solubilized and transferred to vials for counting internalized radioactivity. The points represent the means±s.e.mean (vertical lines) of three separate determinations.

the ligand off quickly without damaging the cells and whilst preventing further internalization of ligand. In this study, we have shown that under normal cellular conditions (i.e. in the presence of GTP and NaCl) 90% of [^{125}I]-BIM-23027 bound to

Table 2 Inhibition of internalization of 0.2 nM [^{125}I]-BIM-23027 in Neuro2A cells by non-radiolabelled analogues of SRIF

Ligand	*pIC_{50} Inhibition of internalization*	*pIC_{50} Inhibition of membrane binding**
BIM-23027	8.00±0.10	–
SRIF-14	7.82±0.15	9.9
SRIF-28	7.11±0.11	9.7
L-362855	6.86±0.06	9.6
BIM-23056	6.04±0.08	7.1

Cells were incubated with both radioligand and non-radiolabelled analogues for 10 min at 37°C and then washed with pH5.0 buffer for 10 min at 15°C to remove surface-bound ligand. In all cases, the maximum inhibition of internalization was indistinguishable from the non-specific internalisation. Values are the mean±s.e.mean of three separate determinations. *Data from Koenig *et al.* (1996a).

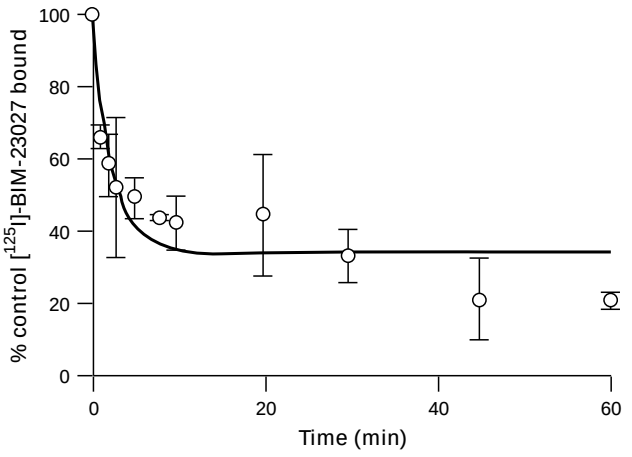

Figure 5 Time course of the removal of surface-bound [^{125}I]-BIM-23027 from ATP-depleted Neuro2A cells. Cells were pretreated with a combination of 50 nM antimycin and 50 mM 2-deoxyglucose for 30 min at 37°C to deplete intracellular ATP. Antimycin and 2-deoxyglucose were also included in subsequent incubations. After incubation with radioligand, the cells were washed for varying amounts of time with pH 7.0 buffer. Line shown is the best fit to a single exponential decay with rate constant 0.41 min^{-1}, and asymptote 33.7%. The points represent the means±s.e.mean (vertical lines) of three separate determinations.

cell membrane homogenates dissociated after a 10 min acid wash at 15°C, whilst 83% dissociated at 4°C within 10 min. Furthermore, internalization of [^{125}I]-BIM-23027 was dramatically decreased at 15°C (Figure 3). It was important to perform the washing step at reduced temperature, since internalization and recycling events are slowed dramatically at temperatures below 20°C (Koenig & Edwardson, 1994b; von Zastrow & Kobilka, 1994). We chose pH 5.0 for the wash

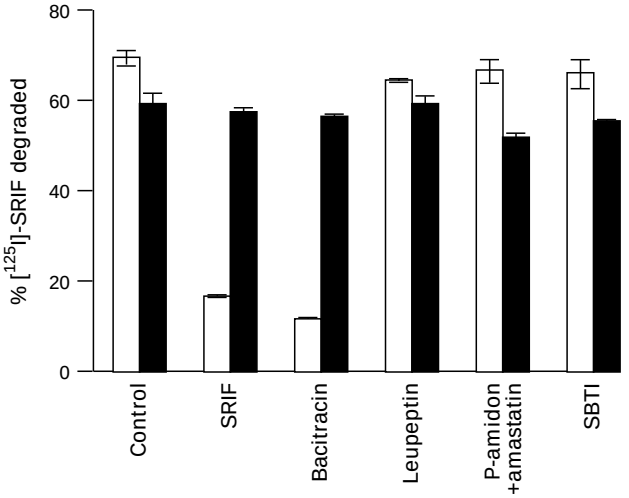

Figure 6 Inhibition of the degradation of [^{125}I]-Tyr^{11}-SRIF-14 by protease inhibitors. Neuro2A cells were incubated with 0.2 nM [^{125}I]-Tyr^{11}-SRIF-14 for 10 min at 37°C in PBS-Ca, Mg containing either 2 μM SRIF-14 or various protease inhibitors, bacitracin (0.16 mg ml^{-1}), leupeptin (8 μg ml^{-1}), phosphoramidon (0.8 μM) and amastatin (8 μM) or soybean trypsin inhibitor (SBTI, 0.8 μg ml^{-1}). The external medium was removed and the internalized radioactivity was extracted by incubation with detergent. Degradation of ligand was assessed through the amount of [^{125}I]-tyrosine separated from intact [^{125}I]-Tyr^{11}-SRIF-14 by Sep-Pak Plus C18 reverse phase minicolumns. Data are the means ± s.e.mean of these separate determinations expressed as the radioactivity eluting with tyrosine as a percentage of total radioactivity in the sample. Open columns represent the radioactivity in the external medium, solid columns represent internalized radioactivity.

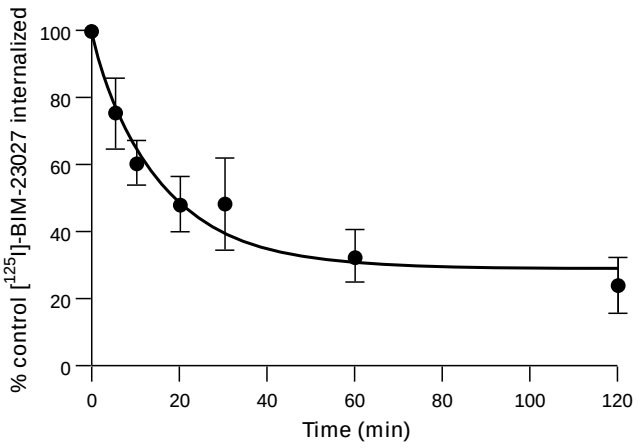

Figure 7 Fate of internalized ligand. Cells were incubated with 0.2 nM [^{125}I]-BIM-23027 for 10 min at 37°C and washed thoroughly with cold pH 5.0 buffer to remove surface-bound ligand then warmed to 37°C with fresh incubation buffer, containing 1 μM SRIF-14, for various times. The amount of recycled ligand in medium is expressed as a percentage of the ligand internalized in the 10 min period.

buffer since, in contrast to more stringent conditions, the cells remain viable after washing at this pH. In addition, more of the ligand appeared to be washed from the cells at pH 5.0 compared to pH 7.4, although this was not statistically significant. Our data also suggest that after internalization, most of the bound ligand is likely to dissociate from the receptor when in the acidic environment of the endosomal compartment. Furthermore, the low affinity at pH 5.0 and 37°C suggests that the ligand is unlikely to reassociate with the receptor. It seems unlikely, therefore, that the receptor will continue to be activated by ligand whilst in endosomes.

Characteristics of [^{125}I]-BIM-23027 internalization

The internalization of [^{125}I]-BIM-23027 was prevented by co-incubation with unlabelled somatostatin analogues. The rank order of potencies of these ligands for inhibition of [^{125}I]-BIM-23027 internalization was similar to that observed for inhibition of binding to Neuro2A membranes, consistent with the involvement of a common receptor. Furthermore, the relative potencies are consistent with interaction at an sst_2 receptor, although the IC_{50} values were about 100 fold higher for inhibition of internalization than the equivalent values for membrane binding. This is perhaps not surprising since membrane binding is performed at equilibrium in the absence of sodium and GTP and presumably involves the high affinity state of the receptor, whereas internalization is performed at 37°C in intact cells prior to equilibrium being reached and may involve binding to the receptor in a low affinity conformation.

A number of other experimental procedures can be used to demonstrate whether or not the mechanism of ligand internalization is consistent with a receptor-mediated event. Thus [^{125}I]-BIM-23027 internalization was reduced by both lowered temperature and ATP depletion (by antimycin and 2-deoxyglucose pretreatment, Hertel *et al*., 1986; Hoover & Toews, 1989). However, reduced temperature (below 20°C) and ATP depletion inhibit many events in the internalization and receptor trafficking process, including translocation of receptors into coated pits (Thatte *et al*., 1996). The presence of hypertonic sucrose (0.5 M) prevents clathrin-coated pit and vesicle formation and is thought to be diagnostic of a clathrin-mediated process (Moore *et al*, 1995). The involvement of a clathrin-mediated process has also been shown for a number of other receptors (e.g. muscarinic, Slowiejko *et al*., 1996; substance P, Garland *et al*., 1993; gastrin releasing peptide, Grady *et al*., 1995). However, in some systems, for example cholecystokinin receptors in Chinese hamster ovary (CHO) cells, receptors can be internalized via both clathrin- and non-clathrin-mediated mechanisms (Roettger *et al*., 1995). We have also found that internalization of [^{125}I]-BIM-23027 in CHO cells containing transfected sst_2 receptors is only partially inhibited by treatment with hypertonic sucrose (J.A. Koenig, unpublished observation).

Dependence of [^{125}I]-BIM-23027 internalization on G-protein coupling

Studies with mutant receptors have provided conflicting evidence as to whether activation of G-proteins is necessary for agonist-induced internalization. Some mutant receptors which show normal G-protein coupling and desensitization do not internalize (e.g. angiotensin$_{1A}$ receptor, Hunyady *et al*., 1994; Thomas *et al*., 1995), whereas there are some mutants which do not couple to G-proteins but can be internalized (e.g. β_2-adrenoceptor, Campbell *et al*., 1991; neurotensin receptor, Chabry *et al*., 1995; thyrotrophin releasing hormone receptor, Gerschengorn & Osman, 1996). There are yet other examples where impairment of G-protein coupling and internalization occur together (e.g. muscarinic m_3 receptor, Thompson *et al*., 1991; muscarinic m_1 receptor, Lameh *et al*., 1992). One approach, when studying G_i/G_0-coupled receptors, is to examine the effect of pertussis toxin on internalization. In this study we have shown that pertussis toxin treatment decreased internalization by approximately half, in agreement with the findings of Hofland *et al*. (1995). This suggests that G-protein coupling may not be essential for internalization but may markedly enhance the efficiency of the internalization process. There are at least two possible explanations for this. One is that pertussis toxin pretreatment decreases high-affinity binding and that a higher concentration of ligand would be needed to internalize the same amount of ligand via interaction with the low affinity conformation. Another possible explanation is that activation of the G-protein amplifies the internalization process which occurs continuously at a low basal rate. An

analogy may be the enhancement of phosphorylation of G protein-coupled receptors by the β-adrenoceptor kinase on activation by $\beta\gamma$ subunits (Premont *et al*., 1995).

Recycling of internalized ligand

The fact that the rate of internalization of ligand decreased with time could result from a number of mechanisms. This phenomenon has been shown with other receptors (e.g., plateau reached within 20 min for the gastrin releasing peptide receptor, Grady *et al*., 1995, and within 90 min for the neuromedin B receptor, Benya *et al*., 1994). In contrast, others have demonstrated a steady rise in the accumulation of internalized ligand (e.g., somatostatin receptor, Hofland *et al*., 1995). A plateau might be reached because the internalization of receptors has depleted the cell surface receptor number. However, this is difficult to reconcile with the low concentration of agonist ligand used in this study (0.2 nM) and did not occur in a study where the number of surface receptors was measured (e.g., gastrin releasing peptide receptor, Grady *et al*., 1995). Another possibility is that the internalized ligand is being recycled and a steady state has been reached. Thyrotropin releasing hormone has been shown to recycle intact with the receptor, although there is some controversy (Gerschengorn & Osman, 1996).

Site of ligand degradation

Sullivan & Schonbrunn (1986) and Presky & Schonbrunn (1989) have shown that somatostatin is degraded at the cell surface and not internalized. There have been other studies where the ligand has been shown to be internalized and then degraded relatively quickly (e.g. Garland *et al*., 1993, 50% substance P degraded in 30 min), although the rate of externalisation of the radioactivity was not determined. Viguerie *et al*. (1987) showed that [^{125}I]-Tyr^{11}-SRIF was degraded in the medium but that internalized ligand was still intact. In the present study, using the Sep-Pak method which detects the presence of [^{125}I]-tyrosine, we found that [^{125}I]-Tyr^{11}-SRIF-14 was rapidly degraded in the extracellular medium; within 10 min at 37°C, 70% of the ligand was degraded. Although this could be almost totally prevented by the inclusion of bacitracin, the viability of the cells was compromised in the presence of this reagent at concentrations greater than 0.04 mg ml^{-1}. The internalized ligand was also substantially degraded, but this could not be prevented by the inclusion of bacitracin. In contrast, only 5% of the radioactivity in [^{125}I]-BIM-23027-treated cells eluted as [^{125}I]-tyrosine, even after a 2 h incubation with cells.

Intracellular versus extracellular binding of [^{125}I]-BIM-23027

It was not possible to characterize the affinity and density of [^{125}I]-BIM-23027 binding sites in intact cells. This probably reflects the low affinity of [^{125}I]-BIM-23027 in the presence of GTP and high non-specific binding. Furthermore, measurement of binding affinity at 37°C was complicated by the internalization of the ligand such that only 20–34% of the ligand was detected at the suface. Hofland *et al*. (1995), showed a similar proportion of surface binding to AtT20 cells after a 30 min incubation at 37°C. AtT20 cells have a receptor/binding site density about 10 fold higher than Neuro2A cells (2.8 pmol mg^{-1} protein in AtT20 cells, Hofland *et al*., 1995; 0.2 pmol mg^{-1} in Neuro2A cells, Koenig *et al*., 1996a).

However, surface binding could be measured when internalization was prevented by ATP depletion. Hertel and colleagues (Hertel *et al*., 1986) and Hoover & Toews (1989) have shown that a combination of 50 nM antimycin and 50 mM 2-deoxyglucose will decrease cellular ATP to below 5% of normal levels. Under these circumstances, the rate of dissociation of [^{125}I]-BIM-23027 was similar to that observed in membranes (Koenig *et al*., 1996a). ATP depletion will not only prevent intracellular trafficking, but will also lead to a depletion of GTP levels since GTP is generated from ATP by the action of nucleoside diphosphate kinase (Blevins *et al*., 1994). This suggests that the surface binding observed in the ATP-depleted cells might reflect the high-affinity state of the receptor. Similar results have been observed with cholecystokinin-receptors (Blevins *et al*., 1994) and opiate receptors (Yabaluri & Medzihradsky, 1995).

In conclusion, we have provided evidence that [^{125}I]-BIM-23027 is internalized in a receptor-dependent, clathrin-dependent manner in Neuro2A neuroblastoma cells. [^{125}I]-Tyr^{11}-SRIF-14 but not [^{125}I]-BIM-23027 is rapidly degraded in the extracellular medium whereas a substantial proportion of internalized [^{125}I]-BIM-23027 is rapidly recycled back to the extracellular medium. Recycling of this radioligand has important implications for the potential usefulness of radiolabelled somatostatin analogues in tumoricidal therapy for somatostatin receptor-expressing tumours.

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