Characterization of ligand binding and processing by bombesin receptors in an insulin-secreting cell line

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Bombesin is a tetradecapeptide which stimulates insulin secretion *in vivo* by isolated islets and by HIT-T15 cells, a clonal line of hamster pancreatic-islet cells. In the present study we have used [¹²⁵I-Tyr⁴]bombesin to characterize bombesin receptors in HIT-T15 cells. [¹²⁵I-Tyr⁴]Bombesin binding was time- and temperature-dependent: maximum binding occurred after 45 min, 90 min and 10 h at 37, 22 and 4 °C respectively. Thereafter, cell-associated radioactivity declined at 37 °C and 22 °C but not at 4 °C. Scatchard analysis of [¹²⁵I-Tyr⁴]bombesin binding measured at 4 °C showed that HIT-T15 cells contain a single class of binding sites ($\cong 85000/\text{cell}$) with an apparent K_d of 0.9 ± 0.11 nM. Structurally unrelated neuropeptides did not compete for [¹²⁵I-Tyr⁴]bombesin binding. However, the relative potencies of bombesin and four bombesin analogues in inhibiting the binding of [¹²⁵I-Tyr⁴]bombesin correlated with their ability to stimulate insulin release. Receptor-mediated processing of [¹²⁵I-Tyr⁴]bombesin was examined by using an acid wash (0.2 M-acetic acid/0.5 M-NaCl, pH 2.5) to dissociate surface-bound peptide from the cells. Following [¹²⁵I-Tyr⁴]bombesin binding at 4 °C, more than 85 % of the cell-associated radioactivity could be released by acid. When the temperature was then increased to 37 °C, the bound radioactivity was rapidly ($t_{1/2} < 3$ min) converted into an acid-resistant state. These results indicate that receptor-bound [¹²⁵I-Tyr⁴]bombesin is internalized in a temperature-dependent manner. In fact, the entire ligand–receptor complex appeared to be internalized, since pretreatment of cells with 100 nM-bombesin for 90 min at 37 °C decreased the subsequent binding of [¹²⁵I-Tyr⁴]bombesin by 90 %. The chemical nature of the cell-associated radioactivity was determined by reverse-phase chromatography of the material extracted from cells after a 30 min binding incubation at 37 °C. Although 70 % of the saturably bound radioactivity was co-eluted with intact [¹²⁵I-Tyr⁴]bombesin, 90 % of the radioactivity subsequently dissociated from cells chromatographed as free iodide. At least some of the degradation of receptor-bound [¹²⁵I-Tyr⁴]bombesin appeared to occur in lysosomes, since chloroquine increased the cellular accumulation of [¹²⁵I-Tyr⁴]bombesin at 37 °C and slowed the release of radioactivity. In summary, our results indicate that the [¹²⁵I-Tyr⁴]bombesin-binding sites characterized here are the receptors which mediate the biological actions of bombesin in HIT-T15 cells and that binding to cell-surface receptors at physiological temperatures is followed by internalization and degradation of the peptide.

INTRODUCTION

Bombesin, a tetradecapeptide originally isolated from the skin of the frog *Bombina orientalis* (Anastasi *et al.*, 1971), is one of a family of structurally homologous amphibian and mammalian peptides which modulate behavioural, metabolic and endocrine processes in mammals (Walsh, 1983). These processes include thermoregulation, pituitary function, gastrointestinal motility and endocrine, as well as exocrine, secretion by the pancreas and gut. In addition, bombesin has been shown to be mitogenic for Swiss 3T3 fibroblasts (Rozengurt & Sinnett-Smith, 1983) and normal human bronchial epithelial cells (Willey *et al.*, 1984). In fact, bombesin-like peptides appear to function as autocrine growth factors for human small-cell lung carcinomas both *in vivo* (Cuttitta *et al.*, 1985) and *in vitro* (Weber *et al.*, 1985). Thus bombesin may be involved in the regulation of cell proliferation as well as in metabolic homeostasis.

High-affinity bombesin-binding sites have been described in several intact cell systems, namely pancreatic acinar cells (Jensen *et al.*, 1978), a clonal line of pituitary cells (Westendorf & Schonbrunn, 1983), two small-cell lung-cancer cell lines (Moody *et al.*, 1985) and Swiss 3T3 fibroblasts (Zachary & Rozengurt, 1985). However, in most of these targets the processing events that occur after bombesin binding have not been investigated.

We have previously shown that bombesin is a potent stimulator of insulin release in HIT-T15 cells (HIT cells), a pancreatic cell line derived from simian-virus-40-transformed hamster pancreatic islets (Santerre *et al.*, 1981; Swope & Schonbrunn, 1984b). Since this cell line retains many of the characteristics of normal pancreatic β -cells, it provides a useful model system for investigating bombesin action. In fact, the effects of bombesin on insulin release in HIT cells parallel those observed both *in vivo* (Schusdziarra *et al.*, 1980; McDonald *et al.*, 1981; Vaysse *et al.*, 1981; Ghatei *et al.*, 1982) and in pancreatic

Abbreviations used: ED₅₀, concentration which causes half-maximal stimulation of insulin release; GRP, gastrin-releasing peptide; Hepes BSS, Hepes-buffered salt solution; HIT cells, the HIT-T15 cell line; ID₅₀, concentration which produces half-maximal inhibition of [¹²⁵I-Tyr⁴]bombesin binding; K_d , equilibrium dissociation constant; R_t , number of receptor sites/cell.

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islets *in vitro* (Kaneto *et al.*, 1978; Ipp & Unger, 1979; Hermansen, 1980). Because pancreatic islets consist of a heterogeneous population of cells, the ability of bombesin to stimulate insulin release in HIT cells first demonstrated that bombesin acts directly on β -cells. In the present study we have characterized high-affinity binding sites for bombesin on HIT cells and examined whether these sites are the functional receptors which mediate the stimulatory effect of bombesin on insulin secretion. Furthermore, since HIT cells become rapidly desensitized to bombesin (Swope & Schonbrunn, 1984b), we have also investigated the processing events that occur after bombesin binding to the cell surface. A preliminary report of some of these experiments has already been presented (Swope & Schonbrunn, 1984a).

EXPERIMENTAL

Materials

Synthetic bombesin, [Tyr⁴]bombesin, ranatensin, litorin, GRP-(1-27)-peptide, GRP-(14-27)-peptide, and substance P were purchased from Peninsula Laboratories, San Carlos, CA, U.S.A.; somatostatin was from Bachem, Torrance, CA, U.S.A. Lactoperoxidase, glucose oxidase, β -D-(+)-glucose, tyrosine, chloroquine and glucagon were purchased from Sigma, St. Louis, MO, U.S.A. Ham's F12 culture medium and sera were purchased from Grand Island Biological Co, Grand Island, NY, U.S.A. Culture plates were obtained from Falcon Labware, Oxnard, CA, U.S.A. Na¹²⁵I (sp. radioactivity 17 Ci/mg) was purchased from ICN, Irvine, CA, U.S.A., and Sep-Pak C₁₈ cartridges were from Waters Associates, Milford, MA, U.S.A.

Methods

Radioiodination of [Tyr⁴]bombesin and tyrosine. [Tyr⁴]Bombesin was radiolabelled with ¹²⁵I by the glucose oxidase/lactoperoxidase method as previously described (Westendorf & Schonbrunn, 1983), except that the reaction was terminated with 0.1% NaN₃ after 45 min. The minimum specific radioactivity of [¹²⁵I-Tyr⁴]bombesin was calculated by assuming complete recovery of peptide after chromatography and ranged from 70 to 280 Ci/mmol in eight separate iodinations.

Tyrosine was radioiodinated with chloramine-T as previously described (Westendorf & Schonbrunn, 1983), except that the tyrosine/¹²⁵I ratio was 10:1. The radiolabelled product co-chromatographed with monoiodotyrosine on Whatman LK6D silica-gel plates in a solvent system of pyridine/3-methylbutan-1-ol/water (7:7:6, by vol.) (R_f = 0.47).

Cell culture. The establishment and properties of the HIT-T15 clonal cell line have been described (Santerre *et al.*, 1981; Swope & Schonbrunn, 1984b). The culture conditions were identical with those used previously to examine insulin secretion (Swope & Schonbrunn, 1984b).

Binding assays. Binding experiments at 4 °C and 22 °C were performed in ambient atmosphere in Hepes BSS [118 mM-NaCl, 4.6 mM-KCl, 0.5 mM-CaCl₂, 1 mM-MgCl₂, 10 mM-glucose, 5 mM-Hepes and bovine serum albumin (1 mg/ml), pH 7.2]. Experiments at 37 °C were performed in CO₂/air (1:19) in the same buffer containing NaHCO₃ (1 mg/ml), pH 7.2. Dishes were washed twice with Hepes BSS and then pre-equilibrated at the appropriate temperature in 1 ml of Hepes BSS for

20 min. The binding reaction was carried out in 1 ml of fresh Hepes BSS containing 50000–100000 c.p.m. of [¹²⁵I-Tyr⁴]bombesin. At the end of the appropriate incubation period, the buffer was aspirated and the dishes were rapidly dipped four times in ice-cold 0.15 M-NaCl. The cells were then dissolved in 0.1 M-NaOH or 1% Nonidet P40 detergent, and cell-associated radioactivity was determined in a γ -radiation counter. Saturable binding was calculated as the difference between amount of [¹²⁵I-Tyr⁴]bombesin bound in the absence (total binding) and presence (non-specific binding) of 100 nM-bombesin and ranged between 90 and 97% of total binding. Unless stated otherwise, the data shown represent saturable binding. To obtain values for the equilibrium dissociation constant (K_d) and the number of receptor sites/cell (R_s), data from saturation binding experiments were plotted by the method of Scatchard (1949) and analysed by linear regression. The ID₅₀ values for bombesin analogues were obtained using a log-logit transformation of competition binding data.

The acid-wash procedure of Haigler *et al.* (1980) was used to determine the cellular distribution of saturably bound [¹²⁵I-Tyr⁴]bombesin. After the binding incubation, cells were washed twice with 1 ml of ice-cold Hepes BSS and treated for 5 min at 4 °C with 1.0 ml of 0.2 M-acetic acid/0.5 M-NaCl, pH 2.5. Cells were then dissolved in 0.1 M-NaOH and the cell-associated radioactivity determined.

Chromatography of ¹²⁵I-, ¹²⁵I-tyrosine and [¹²⁵I-Tyr⁴]bombesin. A modification of the method of Bohlen *et al.* (1980) was used to quantify [¹²⁵I-Tyr⁴]bombesin degradation as previously described (Westendorf & Schonbrunn, 1983). Buffer samples were applied to Sep-Pak C₁₈ mini-columns which had been washed with 10 ml of methanol followed by 10 ml of distilled water. ¹²⁵I-, ¹²⁵I-tyrosine and [¹²⁵I-Tyr⁴]bombesin were sequentially eluted with 5 ml of 0.1% trifluoroacetic acid, 10 ml of 0.1% trifluoroacetic acid in 25% (v/v) methanol, and 10 ml of 0.1% trifluoroacetic acid in 80% methanol respectively. To determine the nature of the radioactivity associated with the cells after the binding of [¹²⁵I-Tyr⁴]bombesin, cells were rinsed twice with ice-cold Hepes BSS and then the bound radioactivity was extracted with 1 ml of 0.1 M-HCl/0.1% BSA. The cell extract was analysed by chromatography on Sep-Pak C₁₈ cartridges as described above. Recovery of radioactive samples from the Sep-Pak columns was previously shown to be 98 ± 6% (Westendorf & Schonbrunn, 1983). All experiments were repeated at least twice, and similar results were obtained in each case.

RESULTS

Kinetics of [¹²⁵I-Tyr⁴]bombesin binding

The time-dependence for [¹²⁵I-Tyr⁴]bombesin binding to HIT cells at different temperatures is shown in Fig. 1. Both the rate of the binding reaction and the maximum amount of [¹²⁵I-Tyr⁴]bombesin bound varied with temperature. At 4 °C, [¹²⁵I-Tyr⁴]bombesin binding reached a plateau at 10 h and remained constant for up to 24 h. In contrast, at 37 °C, cell-associated radioactivity reached a maximum between 30 and 45 min and thereafter declined rapidly. The time course of binding at 22 °C resembled that at 37 °C; however, maximum binding was reached at 90 min, and the decline occurred more slowly. The decrease in [¹²⁵I-Tyr⁴]bombesin binding during prolonged

Fig. 1. Time course of [$^{125}\text{I-Tyr}^4$]bombesin binding

HIT cells [$(3.7 \pm 0.3) \times 10^6$ /well] were incubated with [$^{125}\text{I-Tyr}^4$]bombesin (90 000 c.p.m./ml) at 37 °C (●), 22 °C (○) or 4 °C (×) for the indicated times. Saturably bound [$^{125}\text{I-Tyr}^4$]bombesin was determined as described under 'Methods'. Each point represents the mean result for triplicate wells; the S.E.M. was usually less than 5% of the mean.

incubations at 37 and 22 °C, but not at 4 °C, indicated that either the intact radioactive ligand or a degradation product was released from cells in a temperature-dependent manner and that rebinding of new ligand was inhibited. Since the half-time of [$^{125}\text{I-Tyr}^4$]bombesin in the buffer was approx. 3 h during a 37 °C binding incubation, the observed decrease in binding at this temperature could not be due entirely to the degradation of the trace in the incubation medium. Results presented below support the conclusion that (i) [$^{125}\text{I-Tyr}^4$]bombesin undergoes receptor-mediated endocytosis and degradation and that (ii) receptor binding is decreased by bombesin pretreatment. These processes have been shown to result in transient binding of other hormones and growth factors at physiological temperatures. Furthermore, they complicate analysis of binding data at 37 °C (Wiley & Cunningham, 1981). In order to determine the binding characteristics of the bombesin receptor in the absence of temperature-dependent processing, we carried out subsequent binding experiments at 4 °C. However, we have not been able to demonstrate that a true binding equilibrium exists at this temperature. When HIT cells were allowed to bind [$^{125}\text{I-Tyr}^4$]bombesin at 4 °C, and then incubated at 4 °C in the absence of [$^{125}\text{I-Tyr}^4$]bombesin, essentially none of the bound peptide dissociated from cells during 24 h. Nonetheless, since most of the [$^{125}\text{I-Tyr}^4$]bombesin bound to cells at 4 °C can be released by an acid wash (see below), we would conclude that binding at 4 °C is likely to be reversible even at pH 7.2, albeit very slowly.

Characterization of the [$^{125}\text{I-Tyr}^4$]bombesin-binding sites

The concentration-dependence of [$^{125}\text{I-Tyr}^4$]bombesin binding is shown in Fig. 2. Peptide binding was determined after incubating cells with increasing concen-

Fig. 2. Concentration-dependence of [$^{125}\text{I-Tyr}^4$]bombesin binding

(a) HIT cells [$(1.2 \pm 0.02) \times 10^6$ /well] were incubated at 4 °C for 12 h with the indicated concentrations of [$^{125}\text{I-Tyr}^4$]bombesin (sp. radioactivity = 190 Ci/mmol). Saturable binding was determined as described under 'Methods'. Each point represents the mean result for triplicate wells; the S.E.M. was less than 5%. (b) Scatchard (1949) plot of saturable [$^{125}\text{I-Tyr}^4$]bombesin binding.

trations of [$^{125}\text{I-Tyr}^4$]bombesin at 4 °C for 12 h. The linear Scatchard plot (Fig. 2b) indicates the presence of a single class of non-interacting, high-affinity, binding sites for [$^{125}\text{I-Tyr}^4$]bombesin (Scatchard, 1949). In this experiment the apparent equilibrium dissociation constant (K_d) was calculated as 0.76 nM and the receptor number (R_t) was 120 000 sites/cell. In a second experiment the K_d and R_t values were 0.98 nM and 50 000 sites/cell respectively. The K_d values agreed closely in the two experiments. However, the receptor number was more variable, possibly as a consequence of differences in passage number or cell density.

To examine the structural specificity of the bombesin-binding sites, we determined whether unrelated neuropeptides could inhibit [$^{125}\text{I-Tyr}^4$]bombesin binding. Addition of 10 nM or higher concentrations of glucagon, somatostatin, substance P, epidermal growth factor, vasoactive intestinal peptide, neurotensin, pentagastrin, cholecystokinin octapeptide, secretin, insulin, thyrotropin-releasing hormone and growth-hormone-releasing factor did not affect the binding of [$^{125}\text{I-Tyr}^4$]bombesin

Table 1. Amino acid sequences of bombesin analogues

The peptide sequences shown are from Walsh (1983). The 13 *N*-terminal amino acids of GRP are indicated by '...'. Differences from the bombesin sequence are indicated by underlining of the residues concerned. The 'blanks' in lines 3 and 4 indicate that one has to 'skip' some amino acids in order to achieve maximum homology. Abbreviation used: Glp, pyroglutamic acid (5-oxopyrrolidine-2-carboxylic acid).

Analogue	Sequence
Bombesin	Glp-Gln-Arg-Leu-Gly-Asn-Gln-Trp-Ala-Val-Gly-His-Leu-MetNH ₂
[Tyr ⁴]Bombesin	Glp-Gln-Arg-Tyr-Gly-Asn-Gln-Trp-Ala-Val-Gly-His-Leu-MetNH ₂
Ranatensin	Glp- - - -Val-Pro-Gln-Trp-Ala-Val-Gly-His-Phe-MetNH ₂
Litorin	Glp- - - - -Gln-Trp-Ala-Val-Gly-His-Phe-MetNH ₂
GRP-(1-27)-peptide	...-Met-Tyr-Pro-Arg-Gly-Asn-His-Trp-Ala-Val-Gly-His-Leu-MetNH ₂
GRP-(14-27)-peptide	Met-Tyr-Pro-Arg-Gly-Asn-His-Trp-Ala-Val-Gly-His-Leu-MetNH ₂

Table 2. Potencies of bombesin analogues to compete for [¹²⁵I-Tyr⁴]bombesin binding and to stimulate insulin secretion

The concentration of each bombesin analogue required to produce half-maximal inhibition of [¹²⁵I-Tyr⁴]bombesin binding (ID₅₀) was determined by incubating HIT cells at 4 °C for 12 h with [¹²⁵I-Tyr⁴]bombesin and increasing concentrations of unlabelled analogue. The total amount of [¹²⁵I-Tyr⁴]bombesin bound in the presence of each concentration of unlabelled peptide was determined as described under 'Methods'. In each case the data represent the mean ± S.E.M. for three experiments. The concentration of peptide which causes half-maximal stimulation of insulin secretion (ED₅₀) was measured previously (Swope & Schonbrunn, 1984b). All bombesin analogues elicit the same maximum secretory response.

Analogue	ID ₅₀ (nM)	ED ₅₀ (nM)
Bombesin	1.1 ± 0.35	0.5 ± 0.15
[Tyr ⁴]Bombesin	0.5 ± 0.10	0.5 ± 0.17
Ranatensin	0.8 ± 0.25	1.1 ± 0.08
Litorin	0.7 ± 0.09	0.6 ± 0.05
GRP-(1-27)-peptide	4.5 ± 1.78	2.1 ± 0.61
GRP-(14-27)-peptide	1.4 ± 0.49	0.8 ± 0.30

(results not shown). The inability of structurally unrelated peptides to displace [¹²⁵I-Tyr⁴]bombesin demonstrates the specificity of the bombesin-binding site. Furthermore, the previously reported ability of somatostatin to inhibit, and of glucagon to potentiate, the effect of bombesin on insulin release (Swope & Schonbrunn, 1984b) did not result from modulation of bombesin binding.

We used four bombesin analogues to determine the structural requirements for the binding of bombesin-like peptides. As shown in Table 1, these peptides differ from bombesin both in chain length at the *N*-terminus and in selective amino acid substitutions. [Tyr⁴]Bombesin is the synthetic analogue used for radiolabelling and receptor-binding studies. Litorin and ranatensin, like bombesin, were originally isolated from amphibian skin and contain the *C*-terminal octapeptide sequence of bombesin with a single substitution (Erspamer, 1980). GRP is a mammalian bombesin analogue which was originally isolated from porcine gut (McDonald *et al.*, 1979), and GRP-(14-27)-peptide is a *C*-terminal fragment of GRP, which

has also been extracted from mammalian intestine (Reeve *et al.*, 1983). Both GRP and GRP-(14-27)-peptide contain the *C*-terminal decapeptide sequence of bombesin with a single substitution and differ from bombesin primarily in their *N*-terminal sequence.

All bombesin analogues completely inhibited saturable [¹²⁵I-Tyr⁴]bombesin binding (results not shown), consistent with their ability to stimulate insulin release to the same extent as bombesin (Swope & Schonbrunn, 1984b). For each analogue the concentrations required to give half-maximal inhibition of [¹²⁵I-Tyr⁴]bombesin binding and half-maximal stimulation of insulin release correlated (Table 2). Both the ID₅₀ and ED₅₀ values for GRP were significantly higher than those for bombesin (*P* < 0.01; Duncan's Multiple Range Test). The four short peptides were equipotent with bombesin in competing for [¹²⁵I-Tyr⁴]bombesin binding and stimulating insulin release. Clearly, the *C*-terminal octapeptide sequence of bombesin, which is found in litorin, is sufficient for high-affinity binding and receptor activation. The fact that the biological potencies of the five peptides examined agreed well with their apparent binding affinities indicates that this binding site is the receptor which mediates the secretory response of HIT cells to bombesin.

Receptor-mediated processing of [¹²⁵I-Tyr⁴]bombesin

The time course of [¹²⁵I-Tyr⁴]bombesin binding to HIT cells (Fig. 1) suggested that temperature-dependent ligand processing occurred. Therefore we examined directly the receptor-mediated processing of [¹²⁵I-Tyr⁴]bombesin and determined whether ligand-induced receptor down-modulation was a possible mechanism for the desensitization to bombesin which had been observed in HIT cells (Swope & Schonbrunn, 1984b). To analyse the intracellular distribution of cell-associated [¹²⁵I-Tyr⁴]bombesin we used an acid wash which removes surface-bound ligand and allows quantification of internalized peptide (Haigler *et al.*, 1980). After the binding of [¹²⁵I-Tyr⁴]bombesin to HIT cells for 4 h at 4 °C, 74 ± 8 % (mean ± S.E.M., *n* = 3) of the cell-associated radioactivity could be dissociated by acid. However, 3 min after increasing the temperature to 37 °C, only 30 % of the total cell-associated radioactivity could be removed by the low-pH wash (Fig. 3). Thus 67 % of the surface-bound peptide became resistant to acid dissociation within 3 min (Fig. 3). These data indicate that, after binding to a plasma-membrane receptor, [¹²⁵I-Tyr⁴]bombesin is rapidly internalized at 37 °C.

Poly peptide ligands usually undergo degradation after

Fig. 3. Cellular distribution of bound [¹²⁵I-Tyr⁴]bombesin

HIT cells [(0.7 ± 0.01) × 10⁶/well] were incubated with [¹²⁵I-Tyr⁴]bombesin (100 000 c.p.m./ml) and 0.5 nM unlabelled bombesin at 4 °C for 4 h, at which time the binding of this concentration of bombesin reached a plateau. The cells were then rapidly washed with cold buffer, incubated at 37 °C in 1 ml of Hepes BSS for the indicated time and then divided into two groups. In the first group, total cell-associated radioactivity was determined as described under 'Methods' (○). The second group was treated with 1 ml of 0.2 M-acetic acid/0.5 M-NaCl at 4 °C to remove surface-bound peptide, and the radioactivity which remained cell-associated was then measured (●). The data are means ± S.E.M. for three wells.

receptor-mediated endocytosis. Therefore we next determined whether receptor-bound [¹²⁵I-Tyr⁴]bombesin was degraded by HIT cells (Table 3). After binding [¹²⁵I-Tyr⁴]bombesin at 37 °C for 30 min, the cells were washed and either extracted immediately or incubated with fresh buffer for an additional 2 h at 37 °C to allow dissociation of the bound peptide. After the binding incubation, 66 % of the cell-associated radioactivity chromatographed as intact [¹²⁵I-Tyr⁴]bombesin on reverse-phase minicolumns

(Table 3). However, essentially all of the radioactivity released from the cells during the subsequent 2 h dissociation incubation at 37 °C chromatographed as free ¹²⁵I⁻ (Table 3). The observation that most of the released radioactivity chromatographed as free iodide indicates that HIT cells contain a deiodinating activity which removes ¹²⁵I⁻ from either ¹²⁵I-tyrosine or [¹²⁵I-Tyr⁴]bombesin. Indeed, when HIT cells were incubated directly with ¹²⁵I-tyrosine, free ¹²⁵I⁻ began to accumulate in the medium within minutes. Thus HIT cells contain a deiodinase which can act on iodotyrosine. Although our results do not exclude the possibility that iodide is removed directly from [¹²⁵I-Tyr⁴]bombesin, they are consistent with the conclusion that [¹²⁵I-Tyr⁴]bombesin is degraded by HIT cells and the ¹²⁵I-tyrosine formed as a result is subsequently deiodinated.

Peptides which undergo receptor-mediated internalization are degraded primarily within lysosomes (Gorden *et al.*, 1982). In order to investigate the site of [¹²⁵I-Tyr⁴]bombesin degradation, we used chloroquine, which increases the pH of intracellular acidic compartments, including lysosomes, and thereby inhibits the activity of lysosomal proteinases (Lie & Schofield, 1973). The effect of chloroquine on the rate of release of radioactivity from HIT cells was determined by first incubating the cells with [¹²⁵I-Tyr⁴]bombesin at 4 °C in the presence or absence of 200 μM-chloroquine, and then incubating them for various periods of time at 37 °C in the absence of peptide to allow the prebound ligand to be processed. In the absence of chloroquine, cell-associated radioactivity decreased with first-order kinetics, and a half-time of 20 ± 2 min (mean ± S.E.M., *n* = 6). Release of radioactivity in the presence of chloroquine was also first-order; however, the half-time was increased to 64 ± 1 min (mean ± range, *n* = 2). Methylamine (20 mM), another lysosomotropic agent, and leupeptin (500 μg/ml), an inhibitor of the lysosomal proteinase cathepsin B, gave similar results (results not shown). In contrast with its large effect on the release of iodide from [¹²⁵I-Tyr⁴]bombesin, chloroquine did not significantly alter the rate of deiodination of ¹²⁵I-tyrosine. The percentage of cell-associated ¹²⁵I-tyrosine released as free iodide during a 30 min incubation at 37 °C was 77.4 ± 5.5 % and

Table 3. Degradation of [¹²⁵I-Tyr⁴]bombesin

HIT cells [(5.1 ± 0.5) × 10⁶/well] were incubated for 30 min at 37 °C with [¹²⁵I-Tyr⁴]bombesin (100 000 c.p.m./well). The cells were then washed with Hepes BSS at 22 °C and either extracted with 1.0 ml of 0.1 M-HCl/0.1 % BSA ('cell extract') or incubated at 37 °C in 1 ml of fresh Hepes BSS for an additional 2 h, at which time the buffers were collected ('medium'). Samples were analysed by chromatography on reverse-phase minicolumns as described under 'Methods'. Fractions I, II and III were eluted with 0.1 % trifluoroacetic acid, 0.1 % trifluoroacetic acid/25 % methanol and 0.1 % trifluoroacetic acid/80 % methanol respectively. Data represent means ± S.E.M. for three experimental samples and the value for single standard samples. The values in parentheses show the radioactivity in each fraction as a percentage of the total radioactivity eluted.

Sample	Radioactivity (c.p.m.)		
	Fraction I	Fraction II	Fraction III
Experimental			
Cell extract	440 ± 23 (27)	120 ± 6 (7)	1090 ± 40 (65)
Medium	1310 ± 29 (90)	70 ± 6 (5)	70 ± 6 (5)
Standards			
[¹²⁵ I-Tyr ⁴]Bombesin	5000 (15)	800 (2)	27 200 (83)
¹²⁵ I-Tyrosine	3300 (12)	24 100 (83)	1500 (5)
¹²⁵ I ⁻	9490 (85)	890 (8)	800 (7)

Fig. 4. Effect of chloroquine on the binding of [$^{125}\text{I-Tyr}^4$]bombesin

HIT cells [$(2.5 \pm 0.1) \times 10^6$ /well] were incubated at 37 °C for 30 min in the presence (○) or absence (●) of 200 μM -chloroquine. Subsequently, [$^{125}\text{I-Tyr}^4$]bombesin (65000 c.p.m./ml) was added and the incubation was continued at 37 °C for the times indicated. Saturably bound [$^{125}\text{I-Tyr}^4$]bombesin was determined as described under 'Methods'. Data are means $\pm$ S.E.M. for triplicate wells.

$71.4 \pm 5.2\%$ (mean $\pm$ range, $n = 2$) in the absence and presence of 200 μM -chloroquine respectively.

The effect of chloroquine on the accumulation of radioactivity by HIT cells during incubation with [$^{125}\text{I-Tyr}^4$]bombesin is shown in Fig. 4. At 15 min, and at each subsequent time examined, the amount of radioactivity associated with the cells was greater in the presence of chloroquine than in its absence. After 6 h, essentially all radioactivity had been released from control cells, whereas chloroquine-treated cells still retained approx. 50% of the maximum cell-associated radioactivity (Fig. 4). In summary, chloroquine both increased the cellular accumulation of radioactivity during incubations with [$^{125}\text{I-Tyr}^4$]bombesin and subsequently slowed the release of its degradation product, $^{125}\text{I}^-$. However, it did not inhibit the deiodination of ^{125}I -tyrosine. These results indicate that [$^{125}\text{I-Tyr}^4$]bombesin, but not ^{125}I -tyrosine, is degraded in an acidic compartment, probably the lysosomes.

Taken together, our data examining the processing of receptor-bound [$^{125}\text{I-Tyr}^4$]bombesin indicate that the peptide initially binds to a cell-surface receptor on HIT cells and, at 37 °C, is then rapidly internalized. The differential effect of chloroquine on the deiodination of [$^{125}\text{I-Tyr}^4$]bombesin and ^{125}I -tyrosine indicates that [$^{125}\text{I-Tyr}^4$]bombesin is first slowly degraded by lysosomal proteinases and is subsequently rapidly deiodinated by a non-lysosomal mechanism. The final radioactive degradation product, iodide, is subsequently extruded into the medium.

Since receptor-mediated endocytosis of peptide ligands usually involves internalization of the receptor as well as the bound peptide, this process often results in a reduction in the number of cell-surface receptors. To determine whether ligand-induced down-modulation of the bombesin receptor occurred, the binding of [$^{125}\text{I-Tyr}^4$]bombesin was measured in HIT cells which had been pretreated with bombesin (Table 4). Incubation with 100 nM-bombesin for 90 min at 37 °C decreased the

Table 4. Effect of bombesin pretreatment on [$^{125}\text{I-Tyr}^4$]bombesin binding

HIT cells [$(3.0 \pm 0.3) \times 10^6$ /well] were incubated for 90 min at 37 °C in the presence or absence of 100 nM-bombesin. The cells were then washed twice, and 1 ml of fresh 4 °C buffer containing [$^{125}\text{I-Tyr}^4$]bombesin (100000 c.p.m.) was added. Saturable binding was determined after a 10 h incubation at 4 °C, as described under 'Methods'. Data represent means $\pm$ S.E.M. for three wells.

Pretreatment	[$^{125}\text{I-Tyr}^4$]Bombesin binding (c.p.m.)
Control	2310 $\pm$ 60
Bombesin	220 $\pm$ 40

subsequent binding of [$^{125}\text{I-Tyr}^4$]bombesin by 90% compared with control cells. This large decrease in binding is unlikely to be due simply to occupancy of cell-surface receptors by unlabelled bombesin, since, at 37 °C, bound peptide is internalized with a half-time of less than 3 min, so that most of the cell-associated bombesin will be inside the cell. Therefore the decrease in [$^{125}\text{I-Tyr}^4$]bombesin binding after bombesin pretreatment indicates that bombesin receptors have been altered. The fact that the decrease in [$^{125}\text{I-Tyr}^4$]bombesin binding was similar in magnitude to the desensitization observed upon bombesin pretreatment of HIT cells (Swope & Schonbrunn, 1984b) suggests that receptor down-regulation may be the mechanism by which desensitization to bombesin is triggered.

DISCUSSION

We have previously shown that bombesin stimulates insulin release by HIT cells in a manner which resembles the response seen *in vivo* (Swope & Schonbrunn, 1984b). In the present study we used this cell line and an iodinated bombesin analogue to characterize, for the first time, bombesin-binding sites in pancreatic β -cells. The identified binding sites appear to be functional receptors for bombesin, since the specific binding of [$^{125}\text{I-Tyr}^4$]bombesin was inhibited by bombesin and a series of analogues with the same potencies as they stimulated insulin secretion.

The bombesin receptor in the hamster-derived HIT cell line showed similar structural specificity to the receptors in GH_4C_1 rat pituitary cells (Westendorf & Schonbrunn, 1983), in two human small-cell lung-cancer cell lines (Moody *et al.*, 1985) and in mouse 3T3 fibroblasts (Zachary & Rozengurt, 1985). However, the specificity of the bombesin receptors in guinea-pig pancreatic acinar cells was somewhat different (Jensen *et al.*, 1978). Additional studies will be necessary to determine whether this difference is due to species- or tissue-specificity of bombesin receptors. At this time there are not enough comparative analogue data available to conclude whether a single type of bombesin receptor is present in the various target cells on which this peptide acts.

Specific desensitization to bombesin has been observed both in HIT cells (Swope & Schonbrunn, 1984b) and in pancreatic acinar cells (Deschodt-Lanckman *et al.*, 1976;

Pandol *et al.*, 1982). Our results indicate that down-modulation of the bombesin receptor, occurring as a consequence of the receptor-mediated endocytosis of bombesin, may provide a mechanism for desensitization in HIT cells. This conclusion is supported by several types of experiments. First, when HIT cells were incubated with [125 I-Tyr 4]bombesin at 4 °C, most of the cell-associated radioactivity was released by low pH. However, upon warming to 37 °C, the bound peptide became resistant to dissociation by acid. This temperature-dependent transformation is consistent with initial binding of [125 I-Tyr 4]bombesin to a plasma-membrane receptor being followed by the rapid internalization of the receptor-bound peptide. Secondly, at 37 °C, saturably bound [125 I-Tyr 4]bombesin was degraded by HIT cells, and only the degradation product, 125 I $^-$, was released into the medium. Furthermore, agents known to inhibit lysosomal proteinases both increased the cellular accumulation of [125 I-Tyr 4]bombesin and decreased the rate at which its degradation product was released. Thus the rate-limited steps involved in [125 I-Tyr 4]bombesin degradation probably occur in lysosomes or a similar acidic compartment. Thirdly, treatment of cells with bombesin for 90 min at 37 °C decreased the subsequent binding of [125 I-Tyr 4]bombesin. This decrease in [125 I-Tyr 4]bombesin binding was due to a decrease in the number of receptors per cell and not to a change in receptor affinity (S. L. Swope & A. Schonbrunn, unpublished work). Since, at 37 °C, receptor-bound ligand is rapidly internalized, the observed decrease in receptor number must reflect receptor down-regulation rather than occupancy of cell-surface receptors by bombesin. These results suggest that the desensitization and loss of binding capacity are related events.

Receptor-mediated processing of [125 I-Tyr 4]bombesin has been examined in two cell types other than HIT cells. Both in the GH $_4$ C $_1$ pituitary-cell line and in pancreatic acinar cells, experiments using an acid wash to remove surface-bound peptide indicated that receptor-mediated endocytosis occurred (Westendorf & Schonbrunn, 1983; Pandol *et al.*, 1982). As in HIT cells, the material bound to pituitary and pancreatic acinar cells at 37 °C was primarily in the form of intact peptide. Thus degradation did not occur concomitantly with internalization. Although receptor-bound [125 I-Tyr 4]bombesin was degraded in both GH $_4$ C $_1$ cells and HIT cells, 125 I-tyrosine rather than 125 I $^-$ was released into the medium by the pituitary-cell line (Westendorf & Schonbrunn, 1983). The nature of the material which dissociated from pancreatic acinar cells was not examined; on the basis of the cell-associated material chromatographing as intact peptide, the authors concluded that [125 I-Tyr 4]bombesin was not degraded. The processing of receptor-bound bombesin has not been studied in cell types in which bombesin acts as a mitogen. However, the data available thus far indicate that the initial events occurring after bombesin binding to cell-surface receptors are similar in a variety of different target cells.

An unexpected observation made in the present study was the generation of free iodide from [125 I-Tyr 4]bombesin. In most systems examined, receptor-mediated degradation of iodinated peptides leads to the formation of 125 I-tyrosine, which is then extruded from cells. In fact, this pattern was observed for the receptor-mediated degradation of [125 I-Tyr 4]bombesin in GH $_4$ C $_1$ pituitary cells (Westendorf & Schonbrunn, 1983). Thus it seemed

most likely that iodide was generated from 125 I-tyrosine after the [125 I-Tyr 4]bombesin had been degraded to amino acids rather than being cleaved directly from the intact peptide. In fact three observations support the former mechanism. First, deiodinating activity has been reported to be associated with the endoplasmic reticulum and plasma membrane, but not with lysosomes (Engler & Burger, 1984). Secondly, HIT cells can deiodinate exogenously added 125 I-tyrosine and release the 125 I $^-$ into the buffer. Thirdly, chloroquine inhibited the rate of release of iodide from 125 I-tyrosine but not from [125 I-Tyr 4]bombesin. Thus a chloroquine-sensitive process was the rate-limiting step in the degradation of the peptide, but not in deiodination. Although deiodinating activity has been reported in non-thyroidal tissues, including kidney, liver, heart, pituitary and brain (Engler & Burger, 1984), it has not previously been identified in the pancreas.

In summary, the [125 I-Tyr 4]bombesin-binding sites we have characterized in HIT cells are functional receptors that appear to be down-regulated by bombesin as a consequence of the internalization of the ligand-receptor complex. As in the case of other peptide hormones and growth factors, these processes are likely to be important in modulating cellular responsiveness to bombesin.

This investigation was supported by research grants from the National Institute of Arthritis, Diabetes, Digestive and Kidney Diseases (DK 32234) and from the William F. Milton Fund. S.L.S. was the recipient of an institutional National Research Service training award (5 T32 GM07306).

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Received 26 February 1987/23 June 1987; accepted 30 July 1987