Characterization of the high-affinity receptors on Swiss 3T3 cells which mediate the binding, internalization and degradation of the mitogenic peptide bombesin

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Bombesin and bombesin-related peptides such as gastrin-releasing peptide (GRP) stimulate DNA synthesis and proliferation of Swiss 3T3 cells in culture. We have used ^{125}I -labelled $[\text{Tyr}^4]\text{bombesin}$ and ^{125}I -labelled GRP to characterize and identify the receptors for these peptides on Swiss 3T3 cells. The binding of ^{125}I - $[\text{Tyr}^4]\text{bombesin}$, which retained full biological activity, was maximal between 20 and 30 min incubation at 37 °C, after which continued incubation led to a decline in cell-associated radioactivity. This decline was markedly slowed by the presence of lysosomal enzyme inhibitors. Specificity of the binding site was indicated by the competitive inhibition of binding by bombesin-related peptides, but not by unrelated peptides and growth factors. Scatchard analysis of binding data indicated a single class of high-affinity receptors. The calculated value for the dissociation constant (K_d) was 2.1 nM and each cell possesses approx. 240 000 receptors. Because $[\text{Tyr}^4]\text{bombesin}$ has no free amino group, ^{125}I -GRP was used in chemical cross-linking studies. When disuccinimidyl suberate was used to covalently couple ^{125}I -GRP to the cells, two major radiolabelled complexes were detected with molecular masses of approx. 80 000–85 000 and 140 000. The binding of ^{125}I - $[\text{Tyr}^4]\text{bombesin}$ to the cells was pH-dependent with maximal binding at pH 6.5–7.5 and effectively no specific binding at pH values below 4.5. At 37 °C, cell-associated ^{125}I - $[\text{Tyr}^4]\text{bombesin}$ quickly became resistant to removal by acidic buffers, suggesting its rapid transfer to an intracellular compartment. However, pre-incubation with unlabelled $[\text{Tyr}^4]\text{bombesin}$ did not induce down-regulation of bombesin receptors as measured by the subsequent binding of ^{125}I - $[\text{Tyr}^4]\text{bombesin}$. In contrast with the Swiss 3T3 cells, specific binding of ^{125}I - $[\text{Tyr}^4]\text{bombesin}$ was not detectable in two cell lines which are biologically unresponsive to bombesin-related peptides.

INTRODUCTION

Peptides of the bombesin family elicit a wide range of physiological responses including smooth-muscle contraction and the release of several peptide hormones such as gastrin, cholecystokinin, prolactin and growth hormone (Rivier *et al.*, 1978; McDonald *et al.*, 1979; Walsh *et al.*, 1979; Ghatei *et al.*, 1982; Westendorf & Schonbrunn, 1982; Minamino *et al.*, 1983, 1984). However, in addition to these acute responses, bombesin and related peptides have been shown to have longer-term stimulatory actions on cell proliferation both *in vivo* and *in vitro*. Repeated administration of bombesin to rats causes pancreatic (Lezoché *et al.*, 1981) and gastric cell (Lehy *et al.*, 1983) hyperplasia. Furthermore, bombesin and its mammalian homologue, gastrin-releasing peptide (GRP), are potent mitogens for cultures of Swiss 3T3 cells (Rozenfurt & Sinnett-Smith, 1983; Corps *et al.*, 1985), normal bronchial epithelial cells (Willey *et al.*, 1984) and cell lines derived from small-cell cancer(s) of the lung (SCCL) (Cuttitta *et al.*, 1985; Weber *et al.*, 1985). Since human SCCL and SCCL-derived cell lines are known to synthesize and secrete bombesin-like peptides (Erisman *et al.*, 1982; Sorenson *et al.*, 1982; Moody *et al.*, 1983), it is possible that the peptides may function as autocrine

growth factors involved in the uncontrolled growth of the tumours. This possibility is supported by the finding (Cuttitta *et al.*, 1985) that a monoclonal antibody against bombesin suppressed the growth of SCCL-derived cells.

Swiss 3T3 cells bind radiolabelled GRP (Zachary & Rozenfurt, 1985) and $[\text{Tyr}^4]\text{bombesin}$ (Brown & Laurie, 1986) with high affinity and several recent studies have investigated the biochemical pathways activated when bombesin and related peptides bind to cells. Exposure of Swiss 3T3 cells to bombesin-related peptides causes a rapid stimulation of the hydrolysis of phosphatidylinositol 4,5-bisphosphate (Brown *et al.*, 1984, 1987; Heslop *et al.*, 1986; Sturani *et al.*, 1986; Takuwa *et al.*, 1987) generating inositol 1,4,5-trisphosphate and diacylglycerol, an endogenous activator of protein kinase C (see Berridge & Irvine, 1984; Nishizuka, 1984). Hydrolysis of phosphatidylinositol 4,5-bisphosphate in 3T3 cells is also stimulated by platelet-derived growth factor (Berridge *et al.*, 1984; Chu *et al.*, 1985), but not by insulin, insulin-like growth factor 1, epidermal growth factor (EGF), or transforming growth factor β (Berridge *et al.*, 1985; Besterman *et al.*, 1986; Heslop *et al.*, 1986; Brown & Holley, 1987). The cellular platelet-derived growth factor receptor has been shown to possess inherent tyrosine

Abbreviations used: DSS, disuccinimidyl suberate; EGF, epidermal growth factor; GRP, gastrin-releasing peptide; SCCL, small-cell cancer(s) of the lung; DMEM, Dulbecco's modified Eagle's medium.

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kinase activity and treatment with this growth factor causes a marked increase in total cellular phosphotyrosine levels, and tyrosine phosphorylation both of known substrates and its own receptor. In contrast, we were unable to demonstrate an increased tyrosine phosphorylation of either total cellular protein or known substrates after treatment of Swiss 3T3 cells with bombesin or GRP (Isacke *et al.*, 1986).

Although the binding of ^{125}I -labelled [Tyr⁴]bombesin (Brown & Laurie, 1986) or GRP (Zachary & Rozengurt, 1985) to Swiss 3T3 cells has been reported, the properties of the receptors for bombesin-related peptides have not been fully investigated. In this study we present details on the binding and the apparent internalization and catabolism of ^{125}I -[Tyr⁴]bombesin by Swiss 3T3 cells together with an attempt to identify the cellular receptor for bombesin-like peptides by affinity cross-linking.

EXPERIMENTAL

Materials

Synthetic bombesin, [Tyr⁴]bombesin, GRP and other bombesin-related peptides were obtained from Bachem U.K. Radioisotopes were purchased from Amersham International. Dulbecco's modified Eagle's medium (DMEM), antibiotics, trypsin and newborn-calf serum were from Flow Laboratories and Nunc tissue culture dishes were obtained from GIBCO. Disuccinimidyl suberate (DSS) was purchased from Pierce Chemical Co.

Cell culture

Stock cultures were grown on 9 cm plastic culture dishes (Nunc) in DMEM containing 10% (v/v) newborn-calf serum, 100 units of penicillin/ml and 100 μg of streptomycin/ml. Dishes were incubated at 37 °C in a humidified atmosphere of CO_2/air (1:9). Stock cultures were routinely subcultured before achieving confluence. For experimental use, cells were seeded into 3 cm dishes (2.5×10^4 cells) or 24-well multitrays (1.25×10^4 cells) and used after 6–7 days of growth by which time the cells had formed a confluent and quiescent monolayer as judged by the low (< 2%) proportion of labelled nuclei after a 40 h exposure to [^3H]thymidine.

Peptide iodination

[Tyr⁴]Bombesin was iodinated by the soluble lactoperoxidase procedure (Thorell & Johansson, 1971), and separated from free ^{125}I using a Sep-Pak C_{18} cartridge (Waters) eluted sequentially with 10% and 80% acetonitrile solutions containing 0.1% trifluoroacetic acid. ^{125}I -[Tyr⁴]Bombesin was recovered in the 80% acetonitrile fraction which was reduced in volume under N_2 flux and stored as frozen aliquots at -20 °C. Specific activity of the ^{125}I -[Tyr⁴]bombesin was between 400 and 500 Ci/mmol in nine iodinations. Further analysis of the ^{125}I -[Tyr⁴]bombesin was performed on a Waters h.p.l.c. system using a $\mu\text{Bondapak C}_{18}$ cartridge in a radial compression module. GRP was iodinated and analysed using identical protocols.

Binding of ^{125}I -[Tyr⁴]bombesin to Swiss 3T3 cells

Cells, grown either on 3 cm dishes or 24-well trays, were washed with 1–2 ml of binding medium which consisted of DMEM containing 0.1% (w/v) bovine

serum albumin, 0.1 μM -KI and 50 mM-Bes adjusted to pH 7.0. The cells were incubated with 0.5 ml (trays) or 1 ml (dishes) of binding medium containing ^{125}I -[Tyr⁴]bombesin at the required concentration. After incubation, unbound radioactivity was removed by washing the cells four times with cold (4 °C) phosphate-buffered saline (pH 7.4) containing 0.1% bovine serum albumin and 0.1 μM -KI. The washed cells were solubilized into 1 ml of 0.5 M-NaOH, and cell-associated radioactivity was determined in a gamma radioactivity counter. Non-specific binding was measured in the presence of 200-fold or greater excess of unlabelled [Tyr⁴]bombesin or bombesin. Cell numbers were determined using an electronic cell counter (Coulter).

Identification of putative receptor proteins by affinity cross-linking using DSS

Confluent monolayers of cells were allowed to bind ^{125}I -GRP for 30 min at 37 °C, then cooled to 4 °C and washed twice with cold phosphate-buffered saline (pH 7.4) containing 0.1% bovine serum albumin and 0.1 μM -KI. They were then rinsed and given 1 ml of phosphate-buffered saline (protein-free). DSS was added from a freshly-prepared stock solution in dimethyl sulphoxide to give the required final concentration with a dimethyl sulphoxide concentration of not more than 3% (v/v). Control replicates received dimethyl sulphoxide alone. After 15 min at 4 °C, the cross-linking reaction was quenched by replacement of the solution with cold 150 mM-Tris/HCl (pH 7.4). The cells were gently scraped into suspension in 0.5 ml of phosphate-buffered saline (pH 7.4) and pelleted by centrifugation at 300 g for 10 min at 4 °C. The cell pellet was lysed in 60 μl of cold lysis buffer comprising 10 mM-Tris/HCl (pH 7.4), 10 mM-NaCl, 1.5 mM-MgCl₂ and 1% (v/v) Nonidet P40. Nuclei were removed by centrifugation at 700 g for 10 min at 4 °C. The supernatant was supplemented with the appropriate volume of 5 $\times$ concentrated electrophoresis sample buffer, heated to 100 °C for 4 min and subjected to SDS/polyacrylamide-gel electrophoresis. The gels were stained with Coomassie Blue, destained, dried onto filter paper and exposed with an intensifying screen to Fuji X-ray film for 7–14 days at -70 °C.

RESULTS

Characterization of ^{125}I -labelled [Tyr⁴]bombesin

^{125}I -[Tyr⁴]Bombesin was prepared as described in the Experimental section. The iodinated peptide, which was eluted from the Sep-pak using 80% acetonitrile, retained full biological activity as judged by its ability to stimulate DNA synthesis (Fig. 1a). The dose-response for stimulation of [^3H]thymidine incorporation into quiescent monolayers of Swiss 3T3 cells by the labelled peptide was very similar to that seen for unlabelled [Tyr⁴]bombesin and for bombesin (Fig. 1a; Corps *et al.*, 1985; Heslop *et al.*, 1986). We have analysed the ^{125}I -[Tyr⁴]bombesin by reversed-phase h.p.l.c. and a typical elution profile of ^{125}I radioactivity from a C_{18} column is shown in Fig. 1(b). A single major peak of activity was eluted at an acetonitrile concentration of 47% with a minor peak eluting slightly earlier at 44% acetonitrile. For binding studies, the radiolabelled peptide was used without further purification.

Fig. 1. Properties of ^{125}I -labelled [Tyr⁴]bombesin

(a) Stimulation of DNA synthesis in Swiss 3T3 cells by ^{125}I -[Tyr⁴]bombesin. Cells were grown to confluence over 7 days in DMEM containing 10% calf serum. The confluent quiescent cultures were rinsed with serum-free DMEM and 2 ml of medium, containing [^3H]thymidine (1 $\mu\text{Ci}/\text{ml}$; 1 μM), insulin (1 $\mu\text{g}/\text{ml}$) and the indicated concentration of ^{125}I -[Tyr⁴]bombesin (●), [Tyr⁴]bombesin (■) or bombesin (▲) were added per dish. The cells were incubated at 37 °C for 40 h and then washed and trichloroacetic acid extracted. [^3H]Thymidine incorporated into DNA was determined as described previously (Brown & Blakeley, 1983). Each point represents the mean value for duplicate determinations. [^3H]Thymidine incorporation in the absence of either bombesin or insulin was 3×10^3 c.p.m./dish and stimulation with 10% calf serum produced incorporation of 414×10^3 c.p.m./dish. (b) Reversed-phase h.p.l.c. analysis of ^{125}I -labelled [Tyr⁴]bombesin. A portion of ^{125}I -[Tyr⁴]bombesin ($\sim 130\,000$ c.p.m.) was diluted in 10% acetonitrile/0.1% trifluoroacetic acid and injected onto a C_{18} $\mu\text{Bondapak}$ column (Waters, Radial-Pak, 8 mm $\times$ 10 cm). The elution profile, using a linear gradient of acetonitrile/0.1% trifluoroacetic acid, is indicated. A flow rate of 2 ml/min was maintained throughout and 1 ml fractions were collected and counted for ^{125}I using a gamma counter (LKB Wallac, Bromma, Sweden). Recoveries of applied radioactivity were typically $\sim 90\%$. The elution position of unlabelled [Tyr⁴]bombesin ([Tyr⁴]B) from the same gradient is indicated by the arrow.

Fig. 2. Time course of ^{125}I -[Tyr⁴]bombesin binding to Swiss 3T3 cells at 37 °C and at 4 °C

(a) Cells were rinsed with binding medium and incubated at 37 °C (○) or 4 °C (●) with 1 ml of binding medium containing 2 nM- ^{125}I -[Tyr⁴]bombesin. At the indicated times the cells were washed and cell-associated radioactivity was determined as described in the Experimental section. Each point represents the mean value of duplicate determinations of specific binding. (b) ^{125}I -[Tyr⁴]Bombesin binding was measured at 37 °C as described above in the absence (○) or the presence (●) of 10 mM-NH₄Cl. Each point represents the mean value of triplicate determinations of specific binding.

Time course of ^{125}I -[Tyr⁴]bombesin binding to Swiss 3T3 cells

The time course of ^{125}I -[Tyr⁴]bombesin binding to Swiss 3T3 cells at a ligand concentration of 2 nM is shown in Fig. 2. At 37 °C peptide binding reached a maximum after approx. 30 min. Thereafter, cell-associated radioactivity decreased, and within 4–5 h had reached a level approx. 40% of maximal binding. Agents which inhibit lysosomal enzyme function (e.g. NH₄Cl), caused a substantial slowing in the rate of decrease of cell-associated ^{125}I -[Tyr⁴]bombesin (Fig. 2b). In contrast with the results at 37 °C, ^{125}I -[Tyr⁴]bombesin binding to Swiss 3T3 cells at 4 °C continued to increase for at least 2 h before reaching a plateau value between 30 and 55% of

Fig. 3. Inhibition of ^{125}I -[Tyr⁴]bombesin binding to Swiss 3T3 cells by bombesin-related peptides

Cells were rinsed with binding medium and incubated with 0.5 ml of binding medium containing 1.5 nM- ^{125}I -[Tyr⁴]bombesin and the indicated concentration of bombesin (Δ), [Tyr⁴]bombesin ($\blacksquare$) or GRP ($\bullet$). After a 30 min incubation at 37 °C, the cells were washed and cell-associated radioactivity was determined as described in the Experimental section. Each point represents the mean value for ^{125}I -[Tyr⁴]bombesin binding ($n = 2$ or 3) expressed as a percentage of binding to control cultures not exposed to competing peptide.

the maximal binding at 37 °C (Fig. 2a, and results not shown). Furthermore, at the lower temperature, lysosomal enzyme inhibitors were without effect on the binding curve (results not shown).

Specificity of ^{125}I -[Tyr⁴]bombesin binding

Dose-response curves for the inhibition of ^{125}I -[Tyr⁴]bombesin (1.5 nM) binding to Swiss 3T3 cells by bombesin, [Tyr⁴]bombesin, and GRP are shown in Fig. 3. All three peptides were effective inhibitors of ^{125}I -[Tyr⁴]bombesin binding. Other bombesin-related peptides, of amphibian or mammalian origin, were also able to inhibit binding (results not shown). The ability of the peptides to inhibit binding followed the same potency ranking as that for the stimulation of DNA synthesis: [Tyr⁴]bombesin, bombesin and ranatensin were the most active, being 2–4-fold more potent than GRP, litorin and neuromedin C (GRP 18–27), which in turn were approx. 100-fold more potent than neuromedin B (see Zachary & Rozengurt, 1985, for peptide sequences).

In contrast with the effects of bombesin-related peptides, no non-related peptides, hormones or growth factors were found to inhibit ^{125}I -[Tyr⁴]bombesin binding to these cells. Thus, high (μM) concentrations of

Fig. 4. Binding of ^{125}I -[Tyr⁴]bombesin to Swiss 3T3 cells as a function of ligand concentration

(a) Cells were rinsed with binding medium and incubated with 1 ml of binding medium containing the indicated concentration of ^{125}I -[Tyr⁴]bombesin without ($\circ$) or with ($\bullet$) unlabelled [Tyr⁴]bombesin (5 μM). After a 30 min incubation at 37 °C the cells were washed and cell-associated radioactivity was determined as described in the Experimental section. Each point represents the value for ^{125}I -[Tyr⁴]bombesin binding to a single dish of cells. (b) A Scatchard plot of the specific binding data.

vasopressin, oxytocin, substance P, EGF, platelet-derived growth factor, fibroblast growth factor, insulin and insulin-like growth factors 1 and 2 were without effect on binding (results not shown).

Saturability of ^{125}I -[Tyr⁴]bombesin binding to Swiss 3T3 cells

Specific binding of ^{125}I -[Tyr⁴]bombesin to the cells was saturable (Fig. 4). A Scatchard (1949) plot of the binding data obtained over the concentration range 0.15–18 nM was linear (product moment correlation coefficient, $r = 0.95$) indicating the presence of a single class of non-interacting receptors (Fig. 4). The calculated apparent dissociation constant (K_d) and maximal binding capacity (B_{max}) of the receptors were, respectively, 2.1 nM and 670 pg/10⁶ cells (corresponding to 240 000 receptors per cell assuming a 1:1 molar stoichiometry of ^{125}I -[Tyr⁴]bombesin to receptor). The effect of ^{125}I -[Tyr⁴]bombesin concentration on cellular binding was also measured at 4 °C to prevent cellular processing of ligand. Once again the data produced a straight-line ($r = 0.88$) Scatchard

Fig. 5. Cross-linking of ^{125}I -GRP to Swiss 3T3 cells using DSS

(a) Confluent monolayers of cells were allowed to bind ^{125}I -GRP (20 nM) in the absence (-) or presence (+) of unlabelled bombesin (5 μM). The cells were then treated with DSS at the indicated concentrations, and a cell extract was prepared and analysed by SDS/polyacrylamide-gel electrophoresis using 12.5% acrylamide in the separating gel and 4% acrylamide in the stacking gel as described in the Experimental section. The arrows indicate the positions of the 140000- M_r and 80000-85000- M_r radiolabelled complexes. (b) Similar results were obtained in four separate experiments. However, in one experiment an additional faint, but distinct, band at M_r approx. 110000 (small arrow) was detected.

plot (results not shown) but the B_{max} value (442 pg/ 10^6 cells) was somewhat lower, and the K_d value was somewhat higher (5.2 nM), than the values obtained at 37 °C.

Covalent cross-linking of ^{125}I -[Tyr⁴]bombesin to its receptor on Swiss 3T3 cells

To obtain further information about the structure of the bombesin receptor on Swiss 3T3 cells we attempted to couple ^{125}I -[Tyr⁴]bombesin covalently to its receptor and then analyse the receptor-ligand complex by SDS/polyacrylamide-gel electrophoresis. However, we were unable to demonstrate coupling of the labelled bombesin to a higher molecular mass protein; all the radioactivity applied to the gels ran with the dye front. This failure to cross-link is presumably due to the absence of a free amino group in the peptide. As demonstrated in Fig. 3, the mammalian peptide GRP very effectively competes with ^{125}I -[Tyr⁴]bombesin for binding to Swiss 3T3 cells, and bombesin and [Tyr⁴]bombesin are potent inhibitors of ^{125}I -labelled GRP binding to the cells (Zachary & Rozengurt, 1985). Furthermore, GRP is able to stimulate the same biochemical pathways and biological responses as bombesin and [Tyr⁴]bombesin. These results indicate that the bombesin-like peptides bind to the same receptor on the cells. Since GRP contains a free amino group and is a longer (27 amino acids) peptide than

Fig. 6. Effect of bombesin on the binding and cross-linking of ^{125}I -GRP to Swiss 3T3 cells

Confluent monolayers of cells were allowed to bind ^{125}I -GRP (6 nM) for 30 min at 37 °C in the presence of the indicated concentrations of unlabelled bombesin. The cells were washed, exposed to DSS (0.5 mM) and then scraped into phosphate-buffered saline and pelleted by centrifugation. The points represent ^{125}I -GRP binding to the cell pellets as a function of the concentration of bombesin in the binding medium. The cell pellets were extracted as described in the Experimental section. Extracted proteins were separated by SDS/polyacrylamide-gel electrophoresis using 7.5% acrylamide in the separating gel and 4% acrylamide in the stacking gel. The molecular masses of the specifically labelled complexes are indicated. Similar results were obtained in three separate experiments.

bombesin, we tested the possibility of cross-linking it covalently to the cellular bombesin receptor using DSS. Swiss 3T3 cells were incubated with ^{125}I -GRP to equilibrium, washed free of unbound ligand, and treated with increasing concentrations of DSS. The covalently stabilized complexes were separated by SDS/polyacrylamide-gel electrophoresis and localized by autoradiography. This procedure revealed two major ^{125}I -GRP-protein complexes; one with M_r approx. 140000 and a diffuse band in the M_r range 80000-85000 (Fig. 5a). Incorporation of ^{125}I -GRP into both bands was blocked when binding of ^{125}I -GRP was carried out in the presence of 5 μM unlabelled bombesin (Fig. 5a). Similar results were obtained in four separate experiments but in one experiment an additional faint band of $M_r \sim 110000$ was observed (Fig. 5b). The appearance of the major bands requires cross-linking reagent since neither was visible when cells were treated with dimethyl sulphoxide without DSS, and the bands showed a similar dose-dependency for DSS being detected at a concentration of

Fig. 7. pH dependence of ^{125}I -[Tyr⁴]bombesin binding to Swiss 3T3 cells

A salt solution composed of NaCl (128 mM), KCl (5 mM), MgSO_4 (5 mM) and CaCl_2 (1.2 mM) was buffered at the indicated pH using 40 mM-sodium acetate, -Hepes or -Tris. After rinsing, confluent monolayers of cells were incubated for 30 min at 37 °C in 0.5 ml of the indicated buffered salt solution containing 2 nM- ^{125}I -[Tyr⁴]bombesin without (open bars) or with (shaded bars) unlabelled peptide (1 μM). The cells were washed and cell-associated radioactivity was measured as described in the Experimental section. The bars represent the mean value ($\pm$ S.E.) for triplicate determinations of total binding and the mean value of duplicate determinations for non-displaceable binding.

0.2 mM and apparently maximal by 0.8 mM (Fig. 5a). Even at low concentrations of DSS (0.2 mM), very high M_r material (localized at the origin of the resolving gel) was also labelled, presumably representing aggregates of the ^{125}I -GRP-receptor(s) complex cross-linked to other proteins.

The specificity of ^{125}I -GRP binding to the proteins identified by cross-linking was investigated by studying the competitive effects of low concentrations of bombesin (3–30 nM) on the radiolabelling of the bands. The results show that labelling of both the 80 kDa and the 140 kDa complexes decreased in parallel with the inhibition of ^{125}I -GRP binding to the cells with as little as 3 nM-bombesin causing a substantial reduction in the intensity of labelling (Fig. 6). In this experiment, electrophoresis of cellular proteins was carried out using 7.5% acrylamide in the separating gel compared with 12.5% in the experiments shown in Fig. 5. Under these conditions, the 80 kDa complex forms a wide (but apparently single) band while the 140 kDa complex is resolved into two distinct bands of approx. 140 and 150 kDa.

pH sensitivity of ^{125}I -[Tyr⁴]bombesin binding

Binding of ^{125}I -[Tyr⁴]bombesin to Swiss 3T3 cells is pH-dependent with maximal binding occurring in the range 6.5–7.5 (Fig. 7). At pH 5.5, the level of specific binding was markedly reduced and little, if any, specific binding was detected at pH 4.5 (Fig. 7).

Fig. 8. Cell-bound ^{125}I -[Tyr⁴]bombesin rapidly enters an acid non-releasable compartment at 37 °C

Cells were washed with binding medium and incubated at 37 °C with 1 ml of binding medium containing 1.8 nM- ^{125}I -[Tyr⁴]bombesin. At the indicated times, the cells were washed and 1 ml of cold (4 °C) acetate-buffered salt solution (pH 4.5; see legend to Fig. 5) was added. The dishes were incubated at 4 °C for 30 min and the acidic extract was removed to tubes for ^{125}I counting (acid releasable, $\square$). The cells were solubilized and remaining cell-associated radioactivity (acid non-releasable, $\blacksquare$) was determined as described in the Experimental section. The sum of the acid releasable and acid non-releasable components (total, $\bullet$) is also shown. To correct for non-specific binding, replicate dishes received identical treatment except that the initial binding of ^{125}I -[Tyr⁴]bombesin was carried out in the presence of 5 μM -[Tyr⁴]bombesin. Each point represents the mean value of duplicate determinations.

Internalization and degradation of ^{125}I -[Tyr⁴]bombesin by Swiss 3T3 cells

Failure of ^{125}I -[Tyr⁴]bombesin to bind to cells at low pH resembles the binding behaviour of various other ligands. This property has been used experimentally to distinguish surface-bound extracellular ligand (acid-releasable) from internalized (acid-resistant) ligand (Haigler *et al.*, 1980). After incubation of Swiss 3T3 cells for 4.5 h at 4 °C with 1.8 nM- ^{125}I -[Tyr⁴]bombesin, 84% of specifically bound peptide was released by a 30 min incubation at 4 °C with acetate buffer (pH 4.5). We have compared the levels of acid-releasable and acid-resistant cell-associated ^{125}I -[Tyr⁴]bombesin during binding at 37 °C and found that bound ^{125}I -[Tyr⁴]bombesin rapidly becomes acid-resistant with only 20% of cellular radioactivity being acid-releasable after only a 15 min incubation with the labelled peptide (Fig. 8). This result suggests that at 37 °C, in contrast with 4 °C, the cell-associated ^{125}I -[Tyr⁴]bombesin is rapidly internalized to an intracellular acid-resistant pool. That the internalized ^{125}I -[Tyr⁴]bombesin is degraded is suggested by the observations that cell-associated radioactivity decreases from the maximal level reached after approx. 30 min and that the rate of this decrease is markedly slowed by the presence of inhibitors of lysosomal enzyme function such as NH_4Cl (Fig. 2b).

Fig. 9. Failure of bombesin to down-regulate its receptors on Swiss 3T3 cells

Cells were rinsed with DMEM and incubated for 5 h at 37 °C with binding medium containing the indicated concentration of [Tyr⁴]bombesin. The cells were washed three times with binding medium and incubated for 30 min at 37 °C in binding medium containing a saturating concentration (10 nM) of ¹²⁵I-[Tyr⁴]bombesin with or without 10 μM unlabelled bombesin. The cells were washed and cell-associated radioactivity was measured as described in the Experimental section. Each point represents the mean value (±S.E.) for three separate determinations of specific binding. A second identical experiment gave similar results.

Regulation of ¹²⁵I-[Tyr⁴]bombesin binding capacity of Swiss 3T3 cells

The binding of many ligands to their receptors is associated with an internalization of the ligand-receptor complex and the consequent loss (down-regulation) of receptors from the cell surface. Since our results indicate that ¹²⁵I-[Tyr⁴]bombesin is internalized by Swiss 3T3 cells we have investigated the possible down-regulation of bombesin receptors. Cells were preincubated with increasing concentrations of unlabelled [Tyr⁴]bombesin and then their ability to bind ¹²⁵I-[Tyr⁴]bombesin was measured. Prior exposure to unlabelled peptide in the concentration range 0.6–60 nM, a concentration sufficient to saturate cellular receptors (see Fig. 4), caused no detectable effect on subsequent ¹²⁵I-[Tyr⁴]bombesin binding (Fig. 9). High concentrations (> 600 nM) of peptide in the pre-incubation were required to cause significant effects on binding and even then the maximal inhibition achieved was only 25–30% (Fig. 9, and results not shown).

Binding of ¹²⁵I-[Tyr⁴]bombesin to other cell types

RIE-1 cells are an epithelial cell line established from the rat small intestine (Blay & Brown, 1984, 1985). We have previously reported that bombesin fails to stimulate proliferation of these cells (Blay & Brown, 1986), and consistent with this finding, we have been unable to detect specific displaceable binding of ¹²⁵I-[Tyr⁴]bombesin to the cells (results not shown). NR6 cells are

a variant of Swiss 3T3 cells which were selected for their inability to respond to EGF. The lack of response has been demonstrated to be due to the loss of EGF receptors from the cells (Pruss & Herschman, 1977). NR6 cells showed no evidence of saturable specific binding of ¹²⁵I-[Tyr⁴]bombesin and neither cellular phosphatidylinositol bisphosphate hydrolysis nor DNA synthesis was stimulated by the peptide (results not shown) indicating that the cells lack bombesin receptors as well as EGF receptors. Finally, we examined ¹²⁵I-[Tyr⁴]bombesin binding to NIH/3T3 cells because these cells, like Swiss 3T3 cells, have been reported to show a mitogenic response to bombesin-related peptides (Wakeham *et al.*, 1986). Two different clones were investigated. One showed little evidence of any high-affinity specific binding sites, whereas the other possessed high-affinity binding sites ($K_d \sim 2.5$ nM) but there were only approx. 8000 receptors per cell which is very low compared with the values observed for Swiss 3T3 cells.

DISCUSSION

We have found that Swiss 3T3 cells possess a single class of high-affinity receptors for ¹²⁵I-[Tyr⁴]bombesin. The specificity of these receptors was demonstrated by competitive inhibition by related, but not unrelated, peptides. Binding of the radiolabelled ligand was time-dependent and saturable. The K_d for binding was calculated to be 2.1 nM and each cell has approx. 240 000 receptors. The K_d is close to the concentration (0.5–1 nM) of ¹²⁵I-[Tyr⁴]bombesin required to stimulate DNA synthesis in this cell line. The number of receptors is very high compared with the values previously reported for ¹²⁵I-[Tyr⁴]bombesin binding to pancreatic acini (Jensen *et al.*, 1978), a rat pituitary cell line (Westendorf & Schonbrunn, 1983), or cell lines derived from SCCL (Moody *et al.*, 1985) which have 5000, 4000, and 2000 receptors respectively. Although no previous studies have measured ¹²⁵I-[Tyr⁴]bombesin binding to Swiss 3T3 cells, Zachary & Rozengurt (1985) have examined the binding of ¹²⁵I-GRP to these cells and obtained a value of 100 000 receptors per cell. Thus it appears that these cells do possess an exceptionally high number of receptors for bombesin-related peptides and may, for this reason, prove very valuable in the chemical characterization of these receptors.

Although we were unsuccessful in our attempts to cross-link ¹²⁵I-[Tyr⁴]bombesin to its putative receptor protein, we were able to demonstrate covalent coupling of ¹²⁵I-GRP to cellular proteins. At least two distinctly labelled bands, one at M_r 80 000–85 000 and another at $M_r \sim 140$ 000, were detected on autoradiography of SDS/polyacrylamide gels run under reducing conditions. These same bands were detected using either commercial ¹²⁵I-GRP or peptide iodinated by ourselves. Binding to both bands is specific in that it is strongly inhibited by low concentrations of unlabelled bombesin-related peptides. The relationship of these bands to each other requires further investigation. It is possible that the lower M_r complex is a degradation product formed from a higher M_r protein. However, that this is not the case is suggested by a recent report by Zachary & Rozengurt (1987), who found that an M_r 75 000–85 000 protein of Swiss 3T3 cells could be coupled to ¹²⁵I-GRP using the cross-linking agent ethylene glycolbis(succinimidyl succinate), and that this protein was detected even when

the cells were extracted in the presence of protease inhibitors. In contrast with the results presented here, Zachary & Rozengurt (1987) did not detect any major labelled complex at an M_r above 85000. A possible explanation for this difference is that, whereas in our experiments labelling of cells with ^{125}I -GRP was carried out at 37 °C, Zachary & Rozengurt (1987) employed a reduced temperature (15 °C) in order to restrict peptide internalization. Thus it is possible that the higher M_r band is formed by ^{125}I -GRP binding to a protein in an intracellular compartment, but since we have observed that formation of the higher and lower M_r complexes were equally inhibited by low concentrations of competing peptide, both appear to be due to high-affinity binding.

The high-affinity bombesin receptors demonstrated here appear to mediate the biological responses of the cells to the peptide. Thus, cell lines such as RIE-1 and NR6 which do not possess specific receptors for bombesin-like peptides failed to exhibit any biological responses to these peptides. As mentioned previously, bombesin-like peptides are present throughout the gastro-intestinal tract of mammals (McDonald *et al.*, 1979; Price *et al.*, 1984; Greeley *et al.*, 1986), and hyperplastic effects of the peptide on gastric and pancreatic cells have been reported (Lezoché *et al.*, 1981; Lehy *et al.*, 1983). The absence of bombesin receptors from the RIE-1 cells is particularly interesting since they are an epithelial cell line which was isolated from the rat small intestine (Blay & Brown, 1984) and which may be derived from the proliferative population of crypt cells. These results suggest, therefore, that bombesin-like peptides may not be involved in regulating cell proliferation in the epithelial lining of the small intestine.

The binding of ^{125}I -[Tyr⁴]bombesin to Swiss 3T3 cells was rapid. Maximal binding was reached after a 30 min incubation at 37 °C but continued incubation led to a decline in cell-associated radioactivity. This pattern of binding is very similar to that observed for ^{125}I -[Tyr⁴]bombesin binding to GH₄C₁ cells, a rat pituitary cell line (Westendorf & Schonbrunn, 1983). These authors concluded that the decrease in cell-associated radioactivity was due to receptor-mediated endocytosis and intracellular degradation of the ^{125}I -[Tyr⁴]bombesin. We have obtained evidence that internalization followed by degradation also occurs in the Swiss 3T3 cells, since bound ^{125}I -[Tyr⁴]bombesin rapidly enters an acid non-releasable pool and inhibitors of lysosomal enzyme activity cause a substantial slowing in the decline of cell-associated radioactivity. Furthermore, when cells were incubated with ^{125}I -[Tyr⁴]bombesin for 3 h at 37 °C, we observed the generation of ^{125}I -labelled material which elutes from C₁₈ columns at the same position as [^{125}I]iodotyrosine (results not shown). However, these observations raise the question of why continuing ligand internalization does not maintain the maximal level of cell-associated radioactivity. This could, in principle, be due to a reduction in the number of cell surface receptors if the ^{125}I -[Tyr⁴]bombesin were internalized as a ligand-receptor complex. Paradoxically, however, our results indicate that very little down-regulation of the bombesin receptor occurs (Fig. 9). In addition, we have found (results not shown), that addition of free ^{125}I -[Tyr⁴]bombesin to cells that have undergone a time-dependent reduction in cell-associated radioactivity (e.g. 4 h at 37 °C, Fig. 2) causes a large and rapid rebinding of

^{125}I -[Tyr⁴]bombesin indicating that functional receptors are present on the surface of the cells. We conclude that the decline in cell-associated radioactivity is most likely due to an intracellular degradation of the internalized ligand and that failure to maintain the maximal level of radioactivity is apparently due, not to a fall in cell surface receptors, but to a large reduction in ^{125}I -[Tyr⁴]bombesin able to bind to receptors. Intracellular degradation of the labelled peptide is presumably responsible for much of the observed inactivation. However, whether such processing is able to fully account for the pronounced reduction in the ability of ^{125}I -[Tyr⁴]bombesin to bind to its cellular receptors remains to be established. Catabolism and inactivation of GRP by membrane-bound proteases from pig gastric corpus has previously been demonstrated (Bunnett *et al.*, 1985). The possibility that extracellular proteases may play a part in the processing of bombesin-like peptides by Swiss 3T3 cells requires further study.

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