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Bombesin receptor in membranes from Swiss 3T3 cells

Binding characteristics, affinity labelling and modulation by guanine nucleotides

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Bombesin-like neuropeptides, including mammalian gastrin-releasing peptide (GRP), are potent mitogens for Swiss 3T3 cells. In this study, we have characterized the bombesin receptor in membrane preparations from these cells. Addition of Mg^{2+} during cell homogenization was essential to preserve ^{125}I-GRP binding activity in the resulting membrane preparation. The effect of Mg^{2+} was concentration dependent, with a maximum at 5 mM. Specific binding of ^{125}I-GRP was saturable; Scatchard analysis indicated a single class of high-affinity sites of $K_d = (2.1 \pm 0.3) \times 10^{-10}$ M at 15 °C and $K_d = (1.9 \pm 0.4) \times 10^{-10}$ M at 37 °C, and a maximum binding capacity of 580 ± 50 fmol/mg of protein (15 °C) or 604 ± 40 fmol/mg of protein (37 °C). The kinetically derived dissociation constant was 1.5×10^{-10} M. ^{125}I-GRP binding was inhibited in a concentration-dependent manner by various peptides containing the highly conserved *C*-terminal heptapeptide of the bombesin family, including bombesin, GRP, neuromedin B and the 8–14 fragment of bombesin. In contrast, a variety of structurally unrelated mitogens and neuropeptides had no effect. The cross-linking agent ethyleneglycolbis(succinimidylsuccinate) covalently linked ^{125}I-GRP to a single M_r 75000–85000 protein in membrane preparations of 3T3 cells. Affinity labelling of this molecule was specific and dependent on the presence of Mg^{2+} during membrane preparation. Finally, the non-hydrolysable GTP analogue guanosine-5′-[γ-thio]triphosphate (GTP[S]) caused a concentration-dependent inhibition of ^{125}I-GRP binding and cross-linking to 3T3 cell membranes [concentration giving half-maximal inhibition (IC_{50}) ~ 0.2 μM]. The inhibitory effect was specific (GMP, ATP or ATP[S] had no effect at 10 μM) and was due to an increase in K_d from $(1.7 \pm 0.2) \times 10^{-10}$ M to $(4.3 \pm 0.6) \times 10^{-10}$ M in the presence of 10 μM-GTP[S]. This modulation of ligand affinity and cross-linking implies that the bombesin receptors that mediate mitogenesis in Swiss 3T3 cells are coupled to a guanine-nucleotide-binding-protein signal-transduction pathway.

INTRODUCTION

Regulatory peptides which act as local hormones or neurotransmitters are increasingly implicated in the control of cell proliferation (reviewed in [1]). In particular, bombesin [2] and mammalian peptides structurally related to bombesin including gastrin-releasing peptide (GRP) [3–6] are potent mitogens for Swiss 3T3 cells [7–9], and may act as autocrine growth factors for small cell lung carcinoma [7,10–15]. Before stimulation of DNA synthesis in 3T3 cells, bombesin and related peptides elicit a set of early responses (reviewed in [16]), including enhanced phosphoinositide metabolism [17–20], Ca^{2+} and Na^+ fluxes [18–21], activation of protein kinase C [9,22–26] and induction of the cellular oncogenes c-*fos* and c-*myc* [27,28]

The elucidation of the mechanism of bombesin-stimulated mitogenesis requires the characterization of specific receptors for this peptide in Swiss 3T3 cells. ^{125}I-labelled GRP binds to high-affinity receptors in intact Swiss 3T3 cells [8] and can be cross-linked to an M_r 75000–85000 glycoprotein [29–32]. Subsequently, ^{125}I-GRP is rapidly internalized and degraded by intact Swiss 3T3 cells through a lysosomal pathway [33]. The mechanism of coupling of the bombesin receptor to the signalling systems that trigger mitogenesis remains unclear [27,34,35]. A recent report showed that GTP analogues caused only a small reduction of bombesin binding to 3T3 cell membranes [35]. Hence further experimental work is necessary to define whether a guanine-nucleotide-binding protein [G-protein(s)] is involved in the mechanism of bombesin action in mitogenesis.

The purpose of the present study was to examine in detail the binding of ^{125}I-GRP to membrane preparations from Swiss 3T3 cells. Initial experiments showed that addition of Mg^{2+} during cellular homogenization was necessary to preserve ^{125}I-GRP binding activity in the resulting membrane preparation. Exploiting this observation, we now demonstrate that binding of ^{125}I-GRP is specific, saturable and displaced by unlabelled agonists

Abbreviations used: PBS, phosphate buffered saline; BSA, bovine serum albumin; GRP, gastrin-releasing peptide; EGS, ethyleneglycolbis(succimidylsuccinate); DSS, disuccinimidyl suberate; PAGE, polyacrylamide-gel electrophoresis; VIP, vasoactive intestinal peptide; PDGF, platelet-derived growth factor; FGF, fibroblast growth factor; EGF, epidermal growth factor; PBt_2, phorbol 12,13-dibutyrate; GTP[S], guanosine 5′-[γ-thio]triphosphate; ATP[S], adenosine 5′-[γ-thio]triphosphate; p[NH]ppG, guanosine 5′-[βγ-imido]triphosphate; G-protein, guanine-nucleotide-binding protein; $B_{max.}$, maximal binding capacity; IC_{50}, concentration giving half-maximal inhibition.

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and antagonists. The homobifunctional cross-linking agent ethyleneglycolbis(succimidylsuccinate) (EGS) covalently linked ^{125}I-GRP to a single M_r 75000–85000 protein in Swiss 3T3 membranes. Finally, we determined the effect of guanine nucleotides on the binding and cross-linking of ^{125}I-GRP to assess whether the bombesin receptor from 3T3 cells is coupled to a G-protein.

EXPERIMENTAL PROCEDURES

Materials

Bombesin, GRP, litorin, vasopressin, bradykinin, somatostatin, substance K, substance P, vasoactive intestinal peptide (VIP), epidermal growth factor (EGF), insulin, phorbol 12,13-dibutyrate (PBt_2), bovine serum albumin (BSA), aprotonin, bacitracin, soybean trypsin inhibitor, phenylmethanesulphonyl fluoride and polyethylenimine were purchased from Sigma. GRP(1–16), bombesin(8–14), neuromedin B, [D-Arg^1, D-Pro^2, D-Trp^{7,9}, Leu^{11}] substance P and [D-Arg^1, D-Phe^5, D-Trp^{7,9}, Leu^{11}] substance P were from Bachem Fine Chemicals. EGS and disuccinimidyl suberate (DSS) were obtained from Pierce. GTP, guanosine-5′-[γ-thio]-triphosphate (GTP[S]), GMP, guanosine 5′-[βγ-imido]-trisphosphate (p[NH]ppG), ATP and adenosine-5′-[γ-thio]triphosphate (ATP[S]) were purchased from Boehringer Mannheim. Recombinant platelet-derived growth factor c-*sis* (PDGF), recombinant fibroblast growth factor (FGF), ^{125}I-GRP (1800–2200 Ci/mmol) and ^{125}I-EGF (600–800 Ci/mmol) were from Amersham International. All other reagents were of the highest grade available.

Cell culture

Cultures of Swiss 3T3 cells [36] were maintained in 90 mm Nunc Petri dishes in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, penicillin (100 units/ml) and streptomycin (100 μg/ml) in humidified air/CO_2 (9:1) at 37 °C. For the preparation of membranes, 3×10^6 cells were subcultured into 1850 cm^2 Falcon roller bottles with 200 ml of the same culture medium and were grown to confluence without a change of medium for 6–7 days. The final cell density was 3×10^7 cells/flask.

Membrane preparation

Cultures in roller bottles were washed twice with 150 ml of phosphate-buffered saline (PBS; 0.14 M-NaCl/5 mM-KCl/0.01 M-Na_2HPO_4/1.8 mM-KH_2PO_4, pH 7.2) at room temperature. The cells were then harvested at 4 °C by scraping into ice-cold PBS containing 5 mM-$MgCl_2$, 1 mM-EGTA, 1 mg of bacitracin/ml, 10 μg of aprotinin/ml, 1 mg of soybean trypsin inhibitor/ml and 50 μM-phenylmethanesulphonyl fluoride. All subsequent steps were carried out at 4 °C. The cells were pelleted by centrifugation at 750 *g* for 10 min and resuspended at 5×10^6/ml in solution A containing 50 mM-Hepes, 5 mM-$MgCl_2$, 1 mM-EGTA, 1 mg of bacitracin/ml, 10 μg of aprotinin/ml, 1 mg of soybean trypsin inhibitor/ml and 50 μM-phenylmethanesulphonyl fluoride, adjusted to pH 7.4 with NaOH at 4 °C. Cells were then disrupted using a Dounce Homogenizer (A pestle; 75 strokes). The homogenate was centrifuged at 500 *g* for 10 min to remove nuclear material and intact cells, and the supernatant was centrifuged again at 30000 *g* for 30 min. The resulting pellet, representing a membrane-enriched preparation, was resuspended at a protein concentration of 5–10 mg/ml in solution A and stored in liquid N_2. For experiments, membranes were thawed and diluted to a concentration of 1 mg/ml with solution A. Protein concentrations in the membrane preparations were measured by the method of Bradford [37]. Bovine serum albumin was used as the protein standard.

Receptor binding assay

Binding assays were carried out in a total volume of 100 μl in binding medium containing 50 mM-Hepes, 5 mM-$MgCl_2$, 1 mg of bacitracin/ml and 1% BSA, adjusted to pH 7.4 with NaOH, unless otherwise indicated. The assays contained 25 μg of membrane protein plus 85000–125000 c.p.m. of ^{125}I-GRP (0.5 nM) plus the reagents specified in the individual experiments. Non-specific (non-saturable) binding was determined in the presence of 1 μM of either bombesin or GRP and represented 5–10% of the total binding. Non-specific binding was subtracted from the total binding to obtain specific binding. The membranes were incubated for either 10 min at 37 °C or 30 min at 15 °C as indicated. These conditions provided equilibrium binding.

The binding reactions were terminated at the specified times by rapid filtration on GF/B glass fibre filters (Whatman, 1.0 μm pore size). Each filter was washed five times with 5 ml of PBS containing 1% BSA at 4 °C (15 s total time) using a Millipore filtration apparatus. Before the addition of membranes, the filters were soaked for 24 h in 5% polyethylenimine at 4 °C and washed with 5 ml of PBS containing 1% BSA immediately before use. Identical results were obtained when the assays were terminated by centrifugation for 1 min at 16000 *g* in an MSE microfuge at 4 °C followed by three 1.0 ml washes with PBS containing 1.0% BSA. Radioactivity was determined with a Beckmann γ-counter. The recovery of measurable binding sites in the membrane preparation expressed as a percentage of total sites determined in intact 3T3 cells was approx. 50%. The specific binding activity increased from 204 ± 30 fmol/mg of protein in the intact cells to 564 ± 50 fmol/mg of protein in the membrane preparation.

Chemical cross-linking of ^{125}I-GRP to receptors

Membrane protein (150 μg) prepared from Swiss 3T3 cells as described above was incubated at 30 °C or 15 °C for 10 min or 30 min respectively in 500 μl of cross-linking medium (50 mM-Hepes, 5 mM-$MgCl_2$ and 1 mg of bacitracin/ml, pH 7.4) containing 0.5 nM-^{125}I-GRP and any other reagents as specified in the Results section. BSA was omitted from all solutions used in the cross-linking studies. At the end of the incubation, the membranes were centrifuged at 16000 *g* for 1 min in an MSE microfuge at room temperature. The pellets were then resuspended in cross-linking medium containing 4 mM of the cross-linking reagent (EGS) and incubated at 37 °C or 15 °C for 5 min or 15 min respectively. The reaction was terminated by centrifugation for 1 min followed by one wash with cross-linking medium and centrifugation. Samples were solubilized in 0.20 ml of 2× sample buffer, [0.2 M-Tris/HCl, (pH 6.8)/10% (w/v) glycerol, 6% (w/v) SDS/4% (v/v) β-mercaptoethanol/2 mM-EDTA], immediately heated to 100 °C for 10 min and analysed by one-dimensional electrophoresis.

SDS/polyacrylamide-gel electrophoresis (SDS/PAGE)

Slab gel electrophoresis was performed using 8% acrylamide in the separating gel and 4% in the stacking gel, and 0.1% SDS [38]. After electrophoresis, gels were stained, destained and dried down on to paper for autoradiography with Fuji X-ray film. Dried gels were exposed for 2–4 days at −70 °C. Autoradiograms were scanned using an LKB ultrascan XL densitometer and the incorporation of radioactivity into the M_r 75000–85000 band was quantified using the Ultroscan XL internal digital integrator.

RESULTS

Specific binding of ^{125}I-GRP to Swiss 3T3 cell membranes: requirement for Mg^{2+}

Initial experiments revealed that membrane fractions of Swiss 3T3 cells prepared according to various procedures that were used for other growth factor receptors [39–44] failed to exhibit any consistent specific binding of ^{125}I-GRP. However, addition of Mg^{2+} during the homogenization of the cells as well as during the binding assay resulted in a striking increase in the specific binding of ^{125}I-GRP to membranes. $MnCl_2$ at 2.5 mM only partially substituted for $MgCl_2$ (30% of maximum binding), whereas $CaCl_2$ had no effect. $MgSO_4$ was as effective as $MgCl_2$. Specific ^{125}I-GRP binding as a function of membrane concentration was linear up to 50 μg of protein.

The dependence on Mg^{2+} concentration of ^{125}I-GRP specific binding is shown in Fig. 1. Maximum binding was obtained when Mg^{2+} was present at 5 mM during both the cell homogenization and the binding assay. In contrast, addition of various concentrations of Mg^{2+} to the binding medium using membranes prepared in the absence of this ion increased the specific binding of ^{125}I-GRP to only 25% of the maximum specific binding (Fig. 1). This indicates that Mg^{2+} is required to stabilize the binding activity during the homogenization step.

Specific binding of ^{125}I-GRP (0.5 nM) to membrane fractions of Swiss 3T3 cells prepared in the presence of 5 mM-Mg^{2+} was reduced markedly by addition of either unlabelled bombesin or GRP at 100 nM, but it was not inhibited by other mitogens or neuropeptides (Table 1). These results indicate that ^{125}I-GRP binding to membrane preparations of Swiss 3T3 cells is specific.

Equilibrium and kinetic characteristics of ^{125}I-GRP binding to 3T3 cell membranes

Binding of ^{125}I-GRP to membrane preparations from Swiss 3T3 cells as a function of increasing concentrations of the radiolabelled ligand is shown in Fig. 2. Specific binding of ^{125}I-GRP measured under equilibrium conditions at 15 °C (Fig. 2*a*) was saturable, whereas non-specific binding increased linearly with increasing ligand concentration (results not shown). Scatchard analysis (Fig. 2*a*, inset) of these equilibrium binding data indicated the presence of a homogeneous population of high-affinity binding sites. In six independent experiments performed at 15 °C, the values of K_d and the maximal binding capacity ($B_{max.}$) were $(2.1 \pm 0.3) \times 10^{-10}$ M and 580 ± 50 fmol/mg of protein respectively. Similar experiments carried out at 37 °C (Fig. 2*b*) also showed saturable binding, and the presence of a single class of high-affinity sites with K_d of $(1.9 \pm 0.4) \times 10^{-10}$ M ($n = 4$ experiments) and a $B_{max.}$ of 604 ± 40 fmol/mg of protein (Fig. 2*b*). These values of K_d are in agreement with the value of K_d calculated on the basis of the rate of constants of association and dissociation shown in Table 2.

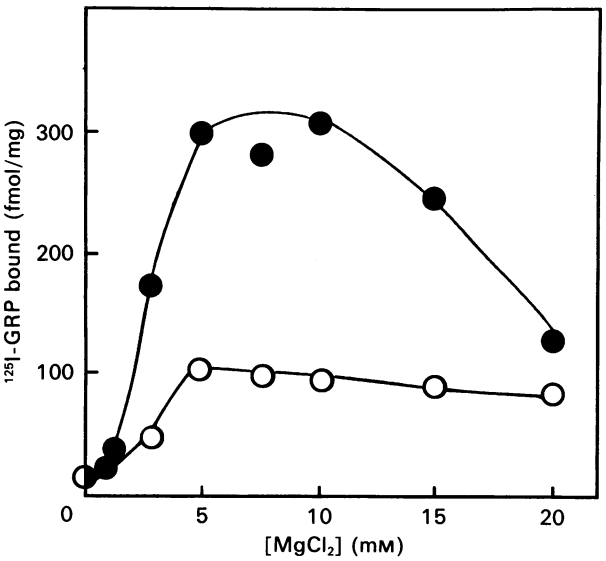

Fig. 1. Specific binding of ^{125}I-GRP to Swiss 3T3 membranes is dependent on $MgCl_2$ concentration

Specific binding of ^{125}I-GRP (0.5 nM) to membrane fractions prepared and assayed at the indicated $MgCl_2$ concentration (●) or prepared in the absence of $MgCl_2$ and assayed at the indicated concentrations of $MgCl_2$ (○). Binding in all cases was measured at 15 °C for 30 min. The results are expressed as the means of triplicate determinations, which varied by less than 5% of the mean. All other details were as described in the Experimental procedures section.

Table 1. Binding of ^{125}I-GRP to Swiss 3T3 membranes is specific

Membrane fractions from Swiss 3T3 cells were incubated in binding medium containing 0.5 nM-^{125}I-GRP and one of the indicated mitogens. After incubation at 37 °C for 10 min, specific binding was determined as described in the Experimental procedures section. The results shown are the means ± S.E.M. ($n = 3$). Similar results were obtained in two other independent experiments.

Addition	Concn.	Binding of ^{125}I-GRP (fmol/mg of protein)
None	–	305 ± 9
Bombesin	100 nM	7 ± 0.2
GRP(1–27)	100 nM	3 ± 0.1
GRP(1–16)	1 μM	299 ± 8
Vasopressin	1 μM	353 ± 10
Bradykinin	1 μM	302 ± 9
VIP	1 μM	353 ± 10
Substance P	1 μM	305 ± 4
Substance K	1 μM	314 ± 5
Somatostatin	1 μM	308 ± 8
PDGF	15 nM	341 ± 9
FGF	6 nM	299 ± 9
EGF	40 nM	305 ± 6
Insulin	1.5 μM	332 ± 6
PBt_2	400 nM	305 ± 9

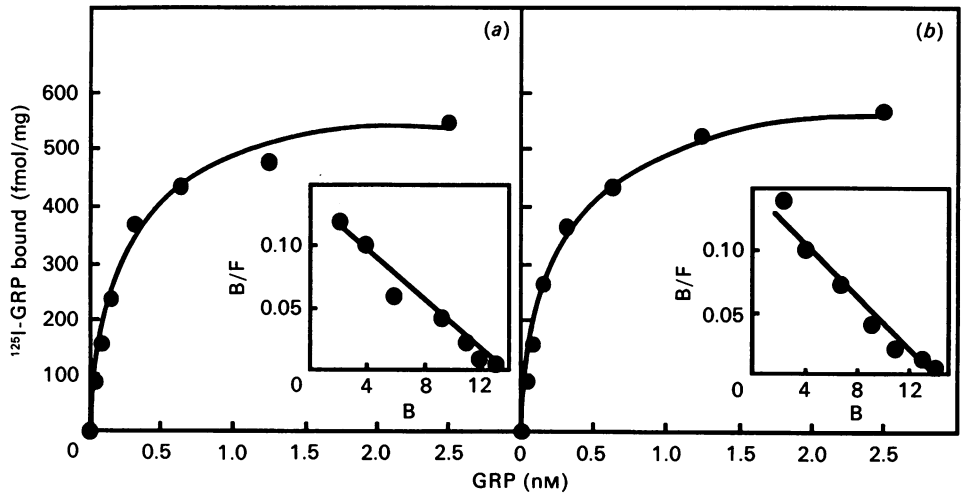

Fig. 2. Analysis of binding as a function of ^{125}I-GRP concentration to the membrane fractions derived from Swiss 3T3 cells at 15 °C and 37 °C

Membranes in 100 μl of binding medium were incubated in the presence of various concentrations of ^{125}I-GRP at either 15 °C (*a*) or 37 °C (*b*). Specific binding was determined after 10 min at 37 °C or after 30 min at 15 °C as described in the Experimental procedures section. Non-specific binding was measured by the addition of at least a 1000-fold excess of unlabelled bombesin or of 1 μM-bombesin for concentrations of ^{125}I-GRP below 1 nM. The insets show Scatchard analysis of the data: bound (B) ^{125}I-GRP is expressed as fmol/25 μg of membrane protein; F, the free ^{125}I-GRP concentration, is expressed in pM.

Table 2. Kinetics of ^{125}I-GRP binding to 3T3 cell membranes

Specific binding of ^{125}I-GRP (0.5 nM) at 15 °C to Swiss 3T3 membrane aliquots (25 μg) was determined at various times as described in the Experimental procedures section. Equilibrium binding was obtained after 15 min and remained constant for 4 h. Dissociation of ^{125}I-GRP was determined by the addition of 1 μM-bombesin to membranes incubated with ^{125}I-GRP (0.5 nM) for 30 min at 15 °C. The specific binding was then determined at various times as described in the Experimental procedures section. The results represent the means ± S.E.M. of three independent experiments. The second-order rate constant of association (k_1) and the first-order rate constant of dissociation (k_2) were determined as described in [50,51].

Constant	Value
Association constant (k_1)	$(0.37 \pm 0.04) \times 10^9$ M^{-1}·min^{-1}
Dissociation constant (k_2)	0.056 ± 0.004 min^{-1}
Equilibrium dissociation constant (K_d) ($K_d = k_2/k_1$)	1.5×10^{-10} M

Bombesin agonists and antagonists inhibit ^{125}I-GRP binding to membrane preparations

The ability of a range of peptides structurally related to GRP to inhibit specific ^{125}I-GRP binding to membrane preparations is shown in Fig. 3. The addition of bombesin, unlabelled GRP, neuromedin B or the (8–14)-amino-acid fragment of bombesin inhibited specific binding of ^{125}I-GRP to the membrane preparation in a concentration-dependent manner. Concentrations giving rise to half-maximal inhibitions (IC_{50}) for bombesin, GRP, neuromedin B and bombesin(8–14) were 1.5, 3.8, 180 and 4000 nM respectively. In contrast, the biologically inactive *N*-terminal fragment of GRP [GRP(1–16)] did not inhibit binding at concentrations up to 100 μM.

The neuropeptide substance P neither inhibits the binding of ^{125}I-GRP to 3T3 cells nor stimulates DNA synthesis in these cells [7,8]. However, substance P antagonists are potent bombesin antagonists in 3T3 cells [45,46]. Fig. 3 also shows that [D-Arg^1, D-Pro^2, D-Trp^{7,9}, Leu^{11}] substance P [8,45] and [D-Arg^1, D-Phe^5, D-Trp^{7,9}, Leu^{11}] substance P [46] inhibited the specific binding of ^{125}I-GRP to membrane preparations of Swiss 3T3 cells. It should be noted that the displacement of ^{125}I-GRP bound to membranes ranges from 10% to 90% over almost two log units of competitor concentration, suggesting a non-cooperative mode of binding for the non-radioactive agonists and antagonists. Furthermore, the IC_{50} values for agonists and antagonists causing inhibition of specific ^{125}I-GRP binding to membranes obtained from Fig. 3 correlated extremely well ($r = 0.98$) with the IC_{50} values for binding to intact cells [8,46].

Cross-linking of ^{125}I-GRP to its receptor in membrane preparations

Membranes from Swiss 3T3 cells prepared in the absence or presence of 5 mM-Mg^{2+} were incubated with ^{125}I-GRP and subsequently treated with the homobifunctional disuccinimidyl cross-linking agent EGS. Analysis by SDS/PAGE followed by autoradiography revealed the presence of a single band migrating with an apparent M_r of 75000–85000 in membranes prepared in the presence of Mg^{2+} (Fig. 4). The formation of this cross-linked complex was completely abolished by addition of a 2000-fold excess of unlabelled bombesin. The M_r 75000–85000 affinity-labelled band was not obtained when the cross-linking reaction was carried out with membrane fractions prepared and assayed in the absence of Mg^{2+}. When membranes prepared without Mg^{2+} were incubated with ^{125}I-GRP in the presence of this ion, the

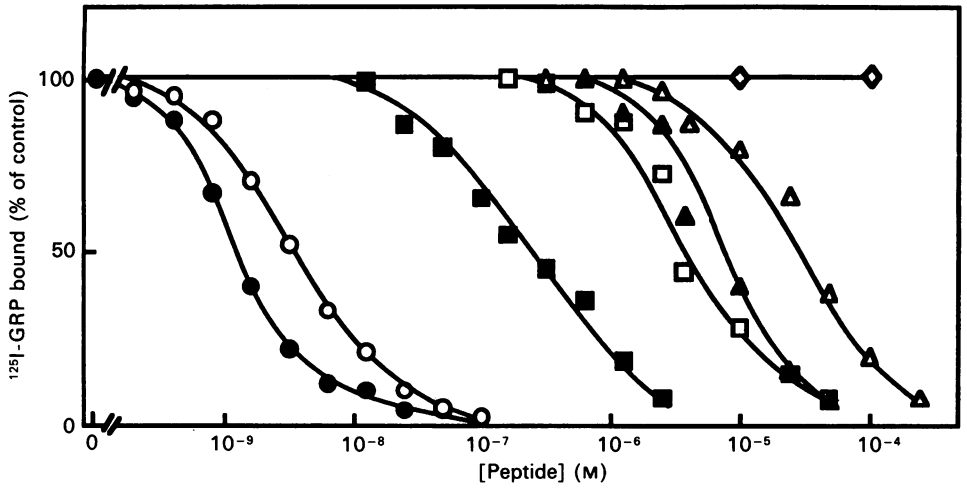

Fig. 3. Effect of various bombesin agonists and antagonists on the specific binding of ^{125}I-GRP to the membrane fraction prepared from Swiss 3T3 cells

Membrane fractions were incubated at 15 °C for 30 min in 100 μl of binding medium containing 0.5 mM-^{125}I-GRP in the absence or presence of the following agonists and antagonists at the concentrations indicated: bombesin (●), GRP (○), neuromedin B (■), bombesin(8–14) (□), GRP(1–16) (◇), [D-Arg^{1}, D-Phe^{5}, D-Trp^{7,9}, Leu^{11}]substance P (▲) and [D-Arg^{1}, D-Pro^{2}, D-Trp^{7,9}, Leu^{11}]substance P (△). The reactions were terminated and specific binding was determined as described in the Experimental procedures section. The results are a composite of two individual experiments and are expressed as a percentage of the control value in each case. The mean control value of specific binding of ^{125}I-GRP was 290±25 fmol/mg of protein (mean±S.E.M., $n = 14$).

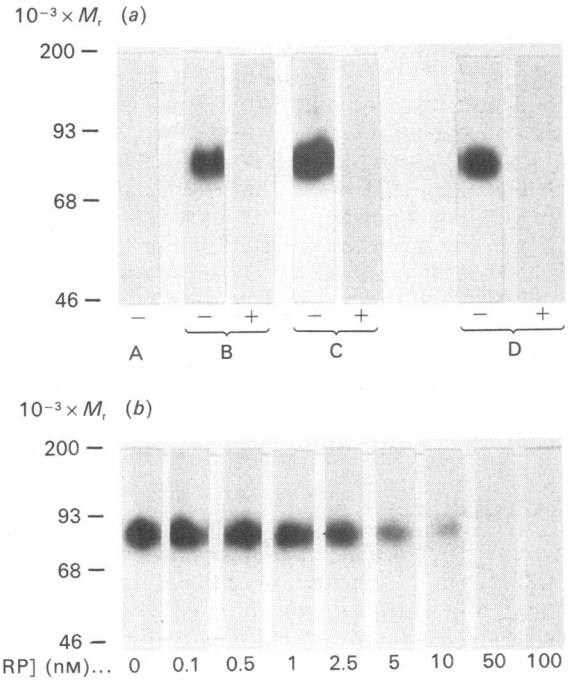

Fig. 4. Affinity labelling of a M_r 75000–85000 membrane protein with the homobifunctional cross-linking agent EGS

(*a*) Dependence on Mg^{2+} for specific affinity labelling. Swiss 3T3 membrane fractions were prepared as described in the Experimental procedures section without $MgCl_2$ (A, B) or with 5 mM-$MgCl_2$ (C). Aliquots (150 μg of membrane protein) were incubated in 500 μl of cross-linking medium without $MgCl_2$ (A) or with 5 mM-$MgCl_2$ (B, C) containing 0.5 nM-^{125}I-GRP, either in the absence (−) or presence (+) of 1 μM-bombesin. After 30 min at 15 °C, the membranes were pelleted by centrifugation, and chemical cross-linking with EGS (4 mM) in the absence (A) or presence (B, C) of 5 mM-$MgCl_2$, was carried out as described in the Experimental procedures section. Similar results were obtained in four independent experiments and also following incubations at 37 °C for 10 min. In other experiments, Swiss 3T3 membranes were purified further by centrifugation on a sucrose solution and then used for chemical cross-linking of ^{125}I-GRP (D). Cells were harvested and homogenized as described in the Experimental procedures section. The supernatant obtained after the removal of whole cells and nuclei was layered on to solution A containing 45% (w/v) sucrose and centrifuged at 9000 *g* for 30 min. The plasma membranes were collected from the interface, diluted 5-fold with solution A and centrifuged at 30000 *g* for 30 min. The pellet was resuspended in the same buffer at a concentration of 1 mg/ml. The membranes (75 μg of protein) were then incubated at 37 °C for 10 min in 500 μl of cross-linking medium with 0.5 nM-^{125}I-GRP in the absence (−) or presence (+) of 1 μM-bombesin. Chemical cross-linking at 37 °C using EGS (4 mM) and analysis by SDS/PAGE were carried out as described in the Experimental procedures section. The specific binding activity of the membranes used in the above experiments measured at 0.5 nM-^{125}I-GRP was 3165 fmol/mg of protein in C and 790 fmol/mg of protein in D. (*b*) GRP inhibits the affinity labelling of the M_r 75000–85000 membrane protein in a concentration-dependent manner. Membrane fractions from Swiss 3T3 cells prepared as described in the Experimental procedures section were incubated in cross-linking medium containing 0.5 nM-^{125}I-GRP at 37 °C for 10 min with unlabelled GRP at the concentrations indicated. Chemical cross-linking using EGS (4 mM) and analysis of the samples by SDS/PAGE were carried out as described in the Experimental procedures section.

labelling of the M_r 75000–85000 band was only 31±4% of that observed with membranes prepared and incubated in the presence of Mg^{2+} (Fig. 4*a*). Thus the formation of the M_r 75000–85000 cross-linked complex correlated

Table 3. Specificity of ^{125}I-GRP affinity labelling of the 75000–85000 protein

Membrane protein aliquots (150 μg) from Swiss 3T3 cells were incubated in cross-linking medium containing 0.5 nM-^{125}I-GRP and the reagents shown. After incubation at 37 °C for 10 min, the membranes were pelleted by centrifugation, and chemical cross-linking was carried out as described in the Experimental procedures section using 4 mM-EGS. The samples were analysed by SDS/PAGE and the autoradiograms were quantified by scanning densitometry as described in the Experimental procedures section. The results shown represent the mean values ± S.E.M. of three independent experiments expressed as a percentage of the level obtained in the absence of additions.

Addition	Concn.	Labelling (% of maximum)
None	–	100
Bombesin	100 nM	5±1
Litorin	100 nM	3±1
GRP(1–27)	100 nM	4±1
Neuromedin B	1 μM	7±2
Bombesin(8–14)	10 μM	5±2
GRP(1–16)	10 μM	102±3
Vasopressin	1 μM	102±1
Bradykinin	1 μM	95±3
Substance P	1 μM	93±2
PDGF	15 nM	104±1
FGF	6 nM	101±1
EGF	40 nM	108±1
Insulin	1.5 μM	113±3
PBt_2	400 nM	111±2

extremely well with the level of specific ^{125}I-GRP binding in the membrane fractions. Mg^{2+} was essential to preserve both specific binding activity and affinity labelling.

EGS promoted cross-linking of ^{125}I-GRP to the M_r 75000–85000 protein in a concentration-dependent manner. The maximum effect was achieved at a concentration of EGS of 4 mM, and a half-maximal effect at 2.5 mM. The cross-linking agent DSS at 2 mM was as effective as EGS in cross-linking ^{125}I-GRP to the M_r 75000–85000 protein (results not shown). Affinity labelling of the M_r 75000–85000 protein was also prominent when using membrane fractions prepared by centrifugation through a sucrose solution (Fig. 4). The level of the M_r 75000–85000 band decreased gradually with increasing concentrations of unlabelled GRP (Fig. 4*b*).

To assess the specificity with which ^{125}I-GRP recognizes the M_r 75000–85000 protein, membrane fractions were incubated with ^{125}I-GRP in the presence of a variety of mitogens and neuropeptides. As shown in Table 3, the cross-linking of ^{125}I-GRP to membrane fractions of Swiss 3T3 cells was markedly inhibited by various peptides structurally related to GRP, including bombesin, litorin and neuromedin B. In contrast, the *N*-terminal fragment of GRP [GRP(1–16)] caused no reduction in the level of the M_r 75000–85000 protein (Table 3). Furthermore, none of the other neuropeptides and growth factors tested reduced the affinity labelling of the M_r 75000–85000 band. These results are in accordance with the finding that the specific binding of ^{125}I-GRP to membrane fractions of Swiss 3T3 cells is also not inhibited by these mitogens.

GTP[S] inhibition of ^{125}I-GRP binding and cross-linking

To determine whether the mitogenic bombesin receptor is coupled to a G-protein, we examined the effect of the non-hydrolysable GTP analogue GTP[S] on the specific binding of ^{125}I-GRP to 3T3 cell membranes. Fig. 5(*a*) shows that GTP[S] inhibited ^{125}I-GRP binding in a concentration-dependent manner; the IC_{50} was 0.17±0.04 μM (mean ± S.E.M.; $n = 6$). At 10 μM, GTP[S] caused a maximal inhibition of ^{125}I-GRP binding of 48±3% ($n = 6$).

Fig. 5(*c*) shows that the level of the cross-linked complex was markedly decreased by GTP[S] in a concentration-dependent manner. The IC_{50} was 0.2 μM and the maximal reduction (54±3%; $n = 5$) was achieved at 10 μM. In other experiments, we found that p[NH]ppG and GTP also inhibited ^{125}I-GRP binding and decreased the amount of the M_r 75000–85000 affinity-labelled band, whereas GMP, ATP and ATP[S], all tested at 10 μM, had no inhibitory effect (Figs. 5*b* and 5*d*).

To define whether the GTP[S]-induced inhibition of ^{125}I-GRP binding was due to a decrease in affinity or in the number of available receptors, the specific binding of ^{125}I-GRP to membranes was measured as a function of increasing concentrations of the radiolabelled ligand in the absence or in the presence of 10 μM-GTP[S]. As shown in Fig. 6, Scatchard analysis of these binding data demonstrated that addition of GTP[S] caused a marked increase in K_d [from $(1.7\pm0.2)\times10^{-10}$ M to $(4.3\pm0.6)\times10^{-10}$ M; $n = 3$], but did not change the $B_{max.}$. Under identical experimental conditions, the binding of various concentrations of ^{125}I-EGF, which binds to a tyrosine kinase receptor not linked to a G-protein, was completely unaffected by 10 μM-GTP[S] (Fig. 6, inset). Kinetic analysis showed that 10 μM-GTP[S] did not change the second-order association rate constant (k_1) but it increased k_2, the rate constant of dissociation (from 0.056±0.004 min^{-1} to 0.13±0.02 min^{-1}; $n = 3$). The results shown in Figs. 5 and 6 clearly demonstrate that the binding and cross-linking of ^{125}I-GRP to the bombesin receptor of Swiss 3T3 cell membranes is regulated by low concentrations of GTP[S] in a specific manner.

DISCUSSION

The characterization of bombesin receptors is an essential step in the elucidation of the molecular basis of the potent mitogenic response initiated by neuropeptides of the bombesin family in cultures of Swiss 3T3 cells. Although binding of ^{125}I-GRP to whole cells has provided valuable information [8,29–31,46], it has also been shown that ^{125}I-GRP is rapidly internalized and subsequently degraded by intact Swiss 3T3 cells through a lysosomal pathway [33]. In many cases, the use of membrane preparations that retain specific binding has provided a useful approach with which to measure precisely the kinetic and equilibrium characteristics of the binding reaction, to identify the binding component(s) and to examine the existence of receptor–G-protein coupling.

Several procedures to prepare membranes from cultured cells and tissues that had succeeded for other growth factor receptors [39–44] failed to preserve ^{125}I-GRP binding activity in membranes from 3T3 cells. In

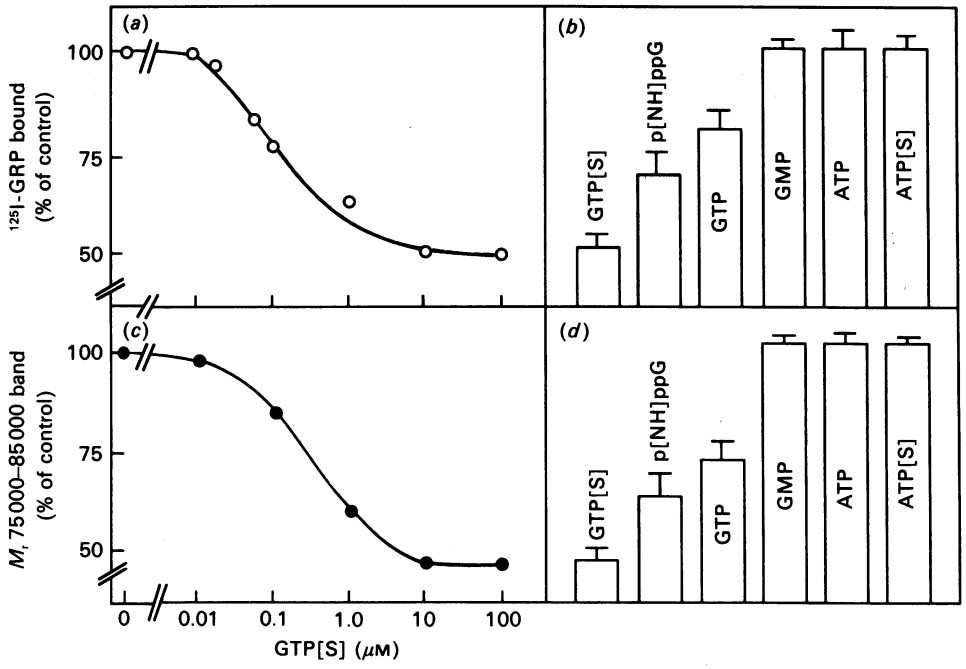

Fig. 5. Guanine nucleotides inhibit ^{125}I-GRP binding and decrease the affinity labelling of the M_r 75000–85000 protein

(*a*, *b*) Effect of nucleotides on ^{125}I-GRP binding. Membrane fractions from Swiss 3T3 cells were incubated at 37 °C for 10 min in 100 μl of binding medium containing 0.1 nM-^{125}I-GRP (15000–25000 c.p.m.) and GTP[S] (○) at the concentrations indicated (*a*) or in the presence of the indicated nucleotides, all at 10 μM (*b*). The reactions were terminated and specific binding was determined as described in the Experimental procedures section. The results are means of three individual experiments (*a*) or the means ± S.E.M. of six independent determinations (*b*). The values are expressed as a percentage of the control values (100% = 102 ± 15 fmol/mg of protein; mean ± S.E.M.; $n = 12$). (*c*, *d*) Effect of nucleotides on the affinity labelling of the M_r 75000–85000 protein. Membrane fractions were incubated with cross-linking medium and 0.5 nM-^{125}I-GRP at 37 °C for 10 min in the presence of GTP[S] (●) at the concentrations shown (*c*) or in the presence of the indicated nucleotides at 10 μM (*d*). Chemical cross-linking using EGS (4 mM) and analysis of the samples by SDS/PAGE were carried out as described in the Experimental procedures section. The values shown are expressed as percentages of the maximum level obtained from scanning densitometry of autoradiograms and are the means of two independent experiments (*c*), or the means ± S.E.M. of five experiments (*d*).

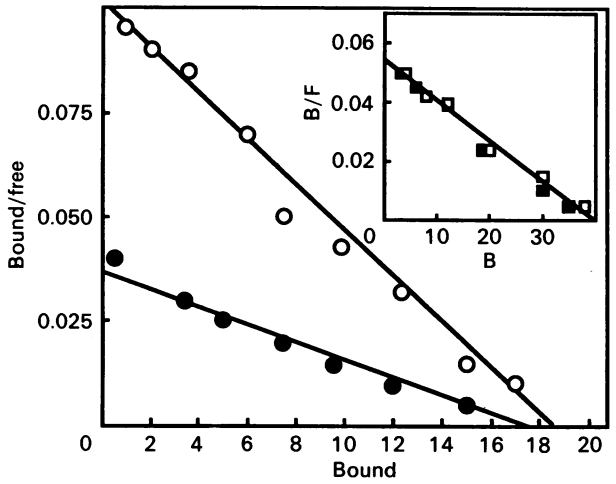

Fig. 6. GTP[S] reduces the affinity of the bombesin receptor for ^{125}I-GRP as determined by Scatchard analysis

Membranes in 100 μl of binding medium were incubated in the presence of various concentrations of ^{125}I-GRP at 37 °C either in the absence (○) or in the presence of GTP[S] (10 μM, ●). Specific binding was determined after 10 min as described in the Experimental procedures section. Non-specific binding was measured by the addition of at least a 1000-fold excess of unlabelled bombesin, or 1 μM-bombesin

the course of these experiments, we found that addition of Mg^{2+} during the preparation of membrane fractions and binding assays resulted in a striking increase of the specific binding of ^{125}I-GRP. The effect was selective; Mg^{2+} could be partially replaced by Mn^{2+}, though not by Ca^{2+}. This effect was not noticed in previous studies using membranes from rat brain [43] or pituitary cells [35]. In our studies the presence of Mg^{2+} during the homogenization of Swiss 3T3 cells was essential to stabilize the ^{125}I-GRP binding activity.

The specificity of the membrane binding sites measured in the present study is supported by the following lines of evidence. (1) Binding of ^{125}I-GRP was not inhibited by a

for concentrations of ^{125}I-GRP below 1 nM. Scatchard analysis of the data is shown: bound ^{125}I-GRP is expressed as fmol/25 μg of membrane protein; the free ^{125}I-GRP concentration is expressed in pM. The inset shows Scatchard analysis of ^{125}I-EGF binding to Swiss 3T3 membranes either in the absence (□) or presence (■) of 10 μM-GTP[S]. Experimental buffers and procedures were identical with those used for ^{125}I-GRP binding to membranes; non-specific ^{125}I-EGF binding was determined using at least a 1000-fold excess of unlabelled EGF. Bound (B) ^{125}I-EGF is expressed as fmol/25 μg of membrane protein; F, the free ^{125}I-EGF, is expressed in pM.

panel of mitogens for Swiss 3T3 cells or by other neuropeptides. (2) Membranes prepared in the presence of Mg^{2+} from 3T6 cells, which neither bind ^{125}I-GRP nor respond to this neuropeptide [7,8], did not exhibit any specific binding activity. (3) Peptides structurally related to GRP containing the highly conserved *C*-terminal heptapeptide of this neuropeptide family, including bombesin, litorin, neuromedin B and the 8–14 fragment of bombesin, inhibited specific binding of ^{125}I-GRP to membrane preparations in a concentration-dependent manner. (4) Two substance P derivatives that function as bombesin antagonists [8,45,46] also inhibited ^{125}I-GRP binding to membranes. The relative potency of various non-radioactive agonists and antagonists in displacing ^{125}I-GRP from membrane preparations correlates extremely well ($r = 0.98$) with the relative abilities of these peptides to inhibit ^{125}I-GRP binding to intact and quiescent Swiss 3T3 cells. Finally, the relative potency of the bombesin agonists to elicit Ca^{2+} mobilization [19,21], ouabain-sensitive Rb^+ uptake [21], protein kinase C-mediated phosphorylation of an acidic M_r 80000 substrate [22,25], inhibition of ^{125}I-EGF binding [9,22], enhancement of cyclic AMP accumulation [47], induction of c-*fos* and c-*myc* expression [28] and stimulation of DNA synthesis in Swiss 3T3 cells [7,8] is the same as their relative abilities to inhibit specific binding of ^{125}I-GRP to membranes derived from 3T3 cells. Taken together, these results indicate that the high-affinity binding sites measured in membrane preparations during this study represent the receptor that mediates the mitogenic effects of the peptides of the bombesin family.

Specific binding of ^{125}I-GRP to membrane preparations of Swiss 3T3 cells was rapid, reversible and consistent with a bimolecular interaction. Scatchard analysis of equilibrium binding data gave a K_d 2.1×10^{-10} M. Using the rate constants of association (k_1) and dissociation (k_2), we calculated an apparent equilibrium dissociation (K_d) of 1.5×10^{-10} M. Hence the kinetically derived equilibrium constant was in agreement with the K_d obtained from equilibrium binding measurements. These results are consistent with a single population of GRP binding sites in membranes of Swiss 3T3 cells. The K_d values measured in membranes are considerably lower (3–10-fold) than the apparent K_d measured previously in intact Swiss or NIH 3T3 cells at 37 °C [8,31]. At this temperature, bound ^{125}I-GRP is rapidly internalized, degraded and then released by intact cells [33], so the concentration dependence with which ^{125}I radioactivity associates with whole cells is likely to differ from that obtained with membranes, in which the binding reaction proceeds in the absence of ligand internalization.

A cell surface glycoprotein of apparent M_r 75000–85000 has been identified by chemical cross-linking as a major component of the bombesin receptor in whole Swiss 3T3 cells [29–31]. However, others observed additional cross-linked bands [32]. It has also been reported that an M_r 115000 protein in Swiss 3T3 cells is phosphorylated at tyrosine in response to bombesin [48], and the possibility that the bombesin receptor is associated with this kinase activity was raised. However, bombesin-stimulated tyrosine kinase was not detected by another laboratory [23], and the M_r of the tyrosine phosphorylated band did not coincide with that of the putative receptor identified by affinity cross-linking in whole cells [29–31]. Hence it was of interest to identify by chemical cross-linking the component(s) that specifically recognizes ^{125}I-GRP in the membrane preparations used in this study. We found that the disuccinimidyl cross-linking agent EGS covalently linked ^{125}I-GRP to an M_r 75000–85000 protein in membrane preparations of Swiss 3T3 cells. Affinity labelling of this component was specific, and was only observed with membranes prepared in the presence of Mg^{2+}. To our knowledge, this is the first time that ^{125}I-GRP has been cross-linked to a protein binding site in a cell-free preparation of any target cell or tissue. A salient feature of our results is that the M_r 75000–85000 affinity-labelled band was the major cross-linked complex detected in the membranes, and appears identical to the major cross-linked complex shown in this [29,30] and other [31] laboratories with 3T3 cells. These findings strongly suggest that the M_r 75000–85000 protein identified in membrane preparations and in whole 3T3 cells is the receptor, or a binding subunit of the receptor.

The availability of membrane preparations that retain specific bombesin receptors is useful in the identification of the signal-transduction mechanism(s) that couples the receptor for these neuropeptides with the generation of intracellular events. A decrease in ligand affinity for receptors produced by added guanine nucleotides is characteristic of a receptor–G-protein interaction [49]. The results presented here demonstrate that the non-hydrolysable GTP analogue GTP[S] caused a specific and concentration-dependent inhibition of ^{125}I-GRP binding and cross-linking to 3T3 cell membranes. The effect is due primarily to an increase in the equilibrium dissociation constant rather than to a decrease in the number of receptors. This modulation of ligand affinity by guanine nucleotides provides evidence that a G-protein couples the mitogenic bombesin receptor with intracellular effector systems.

Evidence for the role of G-proteins in signal transduction pathways can be obtained by assessing the effects of guanine nucleotide analogues on receptor-mediated responses in permeabilized cells. Recently this laboratory reported that bombesin stimulated the phosphorylation of a major protein kinase C substrate in permeabilized cells and that guanosine 5′-[β-thio]diphosphate inhibited this stimulation in a selective manner [25]. These results are consistent with the proposition that the bombesin receptor that mediates mitogenesis in Swiss 3T3 cells is directly linked to a G-protein signal transduction pathway.

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