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Solubilization of the bombesin receptor from Swiss 3T3 cell membranes

Functional association to a guanine nucleotide regulatory protein

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Bombesin and structurally related peptides including gastrin releasing peptide (GRP) are potent mitogens for Swiss 3T3 cells. Here we attempted to solubilize bombesin receptors under conditions in which the ligand (^{125}I-labelled GRP) was prebound to the receptor prior to detergent extraction. We found that ^{125}I-GRP-receptor complexes were solubilized from Swiss 3T3 cell membranes by using the detergents taurodeoxycholate or deoxycholate. These detergents promoted ligand-receptor solubilization in a dose-dependent manner. In contrast, a variety of other detergents including Triton X-100, octylglycoside, CHAPS, digitonin, cholic acid and *n*-dodecyl-β-D-maltoside, were much less effective. Addition of guanosine 5'-[γ-thio]triphosphate (GTPγS) to ligand-receptor complexes isolated by gel filtration enhanced the rate of ligand dissociation in a concentration-dependent and nucleotide-specific manner. Our results demonstrate for the first time the successful solubilization of ^{125}I-GRP-receptor complexes from Swiss 3T3 cell membranes and provide evidence for the physical association between the ligand-receptor complex and a guanine nucleotide binding protein(s).

Signal transduction; Growth control; Neuropeptide

1. INTRODUCTION

Bombesin and structurally related peptides including gastrin-releasing peptide (GRP) are potent mitogens for Swiss 3T3 cells [1,2] and may act as autocrine growth factors for small cell lung carcinoma [3,4]. Prior to stimulation of DNA synthesis in 3T3 cells, bombesin and GRP elicit a set of early molecular responses [5] including enhanced phosphoinositide metabolism, Ca^{2+} and Na^+ fluxes, activation of protein kinase C, enhancement of cAMP accumulation and induction of the cellular oncogenes c-*fos* and c-*myc* (reviewed in [5–7]).

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. ^{125}I-labelled GRP (^{125}I-GRP) binds to high-affinity receptors in intact Swiss 3T3 cells and can be cross-linked to an M_r 75000–85000 glycoprotein [2,8–11]. Subsequently, ^{125}I-GRP is rapidly internalized and degraded by intact Swiss 3T3 cells through a lysosomal pathway [12]. Recently, membrane preparations that retain specific binding have been used to determine kinetic and equilibrium characteristics of the binding reaction, to identify the binding component(s) and to demonstrate modulation of ligand affinity by guanine nucleotides [13]. As yet, however, there is no evidence showing physical association between the bombesin receptor from either Swiss 3T3 cells or any other source to a guanine nucleotide binding regulatory protein(s) (G protein).

The molecular and regulatory characterization of plasma membrane receptors requires a procedure for their solubilization in a functional state. In the present study we describe the solubilization of a ^{125}I-GRP-receptor complex from Swiss 3T3 cell membranes and demonstrate that the dissociation of this complex is sensitive to guanine nucleotides.

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2. MATERIALS AND METHODS

2.1. *Materials*

Bombesin, GRP, digitonin, octylglucoside, Triton X-100, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid (CHAPS), cholic acid, *n*-dodecyl-β-D-maltoside, sodium taurodeoxycholate (TDOC), sodium deoxycholate (DOC), bovine serum albumin (BSA), aprotonin, bacitracin, soybean trypsin inhibitor, phenylmethylsulphonyl fluoride and polyethyleneimine were purchased from Sigma. GTP, guanosine-5'-[γ-thio]triphosphate (GTPγS), GMP, ATP and adenosine-5'-[γ-thio]triphosphate (ATPγS) were purchased from Boehringer Mannheim. ^{125}I-GRP (1800–2200 Ci/mmol) was from Amersham International. All other reagents were of the highest grade available.

2.2. *Methods*

2.2.1. Cell culture

Cultures of Swiss 3T3 cells [14] were maintained in 90 mm Nunc Petri dishes in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, penicillin 100 U/ml, and strep-

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tomycin (100 μg/ml) in humidified 10% CO_2 and 90% air 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.

2.2.2. Membrane preparation

Cultures in roller bottles were washed twice with 150 ml 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 [ethylenebis(oxyethylenenitrilo)]tetraacetic acid (EGTA), 1 mg/ml bacitracin, 10 μg/ml aprotonin, 1 mg/ml soybean trypsin inhibitor and 50 μM phenylmethylsulphonyl 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 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (Hepes), 5 mM $MgCl_2$, 1 mM EGTA, 1 mg/ml bacitracin, 10 μg/ml aprotonin, 1 mg/ml soybean trypsin inhibitor and 50 μM phenylmethylsulfonyl fluoride, adjusted to pH 7.4 with NaOH at 4°C [13]. Cells were then disrupted using a Dounce homogeniser (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 nitrogen. Protein concentration in the membrane preparations was measured by the method of Bradford [15]. BSA was used as the protein standard.

2.2.3. Binding of ^{125}I-GRP to membranes

Swiss 3T3 membranes (50 μg) were incubated at 37°C for 10 min in binding medium (100 μl) containing 0.5 nM ^{125}I-GRP, 30 mM Hepes (pH 7.4), 5 mM $MgCl_2$, 0.25 M sucrose, 1 mg/ml bacitracin and 10 μg/ml aprotonin. The incubation was stopped by rapid vacuum filtration over GF/B filters, presoaked in polyethylenimine (5%) at 4°C. These were then washed with 5 × 5 ml of PBS containing 1% BSA. Under these conditions specific binding was determined as the difference between the amount of ^{125}I-GRP bound in the absence (total binding) and the presence (non-saturable binding) of 10 μM bombesin.

2.2.4. Solubilization of ^{125}I-GRP-receptor complexes

Membranes (0.8–1 mg) were incubated with 1 ml of binding medium at 37°C for 10 min. Following centrifugation at 16000 × *g* for 30 min to remove unbound ^{125}I-GRP, the pellet was resuspended at 4°C in 250 μl of solubilization buffer consisting of 30 mM Hepes (pH 7.4), 5 mM $MgCl_2$, 0.25 M sucrose, 10% glycerol, 1 mg/ml bacitracin, 10 μg/ml aprotonin and the required concentration of detergent. After 30 min at 4°C, the solubilized proteins were separated from non-extractable membrane material by centrifugation for 1 h at 100000 × *g*.

2.2.5. Separation of ^{125}I-GRP from ^{125}I-GRP-receptor complexes

2.2.5.1. *Gel filtration.* ^{125}I-GRP associated with macromolecular components was separated from any free ^{125}I-GRP by gel filtration on a Sephadex G-100 column (20 × 1 cm). The column was equilibrated and eluted at 4°C with 30 mM Hepes, pH 7.4, containing 5 mM $MgCl_2$, 1% BSA and 0.1% of the required detergent. Fractions of 0.5 ml were collected at a flow rate of 7 ml/h. Radioactivity in each case was determined with a Beckman γ-counter. Specific solubilized counts were in each case the difference between the total counts in the fractions representing the complex (typically 12, 13 and 14; Fig. 1) obtained in the absence of bombesin during prelabelling and the total counts of the corresponding fractions from an identical column obtained in the presence of 10 μM bombesin during prelabelling. The percentage of solubilization was then calculated with respect to the total specific binding of ^{125}I-GRP to equivalent amounts of membranes, determined as described previously (binding of ^{125}I-GRP to membranes).

2.2.5.2. *Spun-column chromatography.* In other experiments, ^{125}I-GRP associated with macromolecular components was separated from free ^{125}I-GRP by applying aliquots (50–100 μl) to 1 ml syringes packed with Sephadex G-100 equilibrated in 30 mM Hepes, pH 7.4, 5 mM $MgCl_2$, 1% BSA followed by centrifugation at 1000 × *g* for 1 min. The columns were then washed by centrifugation (1000 × *g*, 1 min) with 200 μl 30 mM Hepes, pH 7.4, 5 mM $MgCl_2$. The radioactivity in the total elution volume was then determined using a Beckman γ-counter.

3. RESULTS AND DISCUSSION

The binding of various hormonal peptides to their corresponding membrane receptors has been shown to stabilize the receptor molecules as well as to induce tight association between the receptor and their respective G proteins [16–20]. Consequently, we attempted to solubilize bombesin/GRP receptors under conditions in which the ligand was prebound to the receptor prior to detergent extraction. Membrane preparations from Swiss 3T3 cells were incubated with 0.5 nM ^{125}I-GRP and then freed of unbound ligand by centrifugation. The membrane pellet was resuspended in a solution containing 0.5% taurodeoxycholate (TDOC) and incubated for 30 min at 4°C. The solubilized material was separated from the non-extractable material by centrifugation for 60 min at 100000 × *g* and chromatographed on a Sephadex G-100 column at 4°C. Fig. 1A shows that a sharp peak of radioactivity was eluted in the void volume while the remaining radioactivity coeluted with free ^{125}I-GRP indicating that only a partial dissociation of the solubilized ligand-receptor complex occurred during the chromatographic separation. This procedure resulted in the solubilization of 46.3 ± 2.7% (n = 10) of the available receptors in the intact membranes. A similar chromatographic profile was obtained when 0.5% deoxycholate (DOC) was used instead of TDOC (Fig. 1B). In each case, the sharp peak of radioactivity eluting in the void volume of the G-100 column was abolished by adding an excess of unlabelled bombesin together with ^{125}I-GRP during the labelling of the membrane. In this case, the radioactivity eluted in the position of free ^{125}I-GRP (Fig. 1A,B).

In other experiments, membranes were treated with TDOC and then the solubilized material was incubated with 1 nM ^{125}I-GRP for 30 min at 37°C prior to chromatography. Fig. 1C shows that no peak of specifically bound radioactivity was eluted in the void volume of the column. These results indicate that TDOC and DOC extracted a ligand-receptor complex formed on intact membranes prior to detergent solubilization and ruled out the possibility that ^{125}I-GRP became bound to non-specific macromolecular components such as mixed micelles present in the elution buffer. Furthermore, when membranes of 3T6 cells, which do not express bombesin receptors [1,2,8], were detergent-solubilized and chromatographed using conditions identical to those depicted in Fig. 1A, no

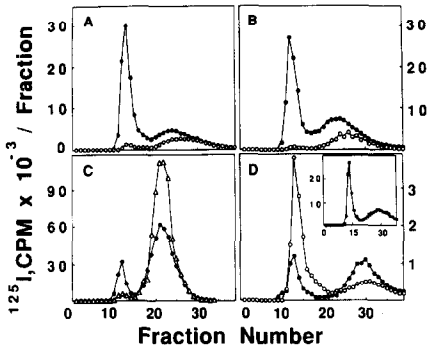

Fig. 1. Gel filtration profiles of solubilized ^{125}I-GRP-receptor complexes (A,B). Swiss 3T3 membranes (0.9 mg) were incubated in binding medium for 10 min at 37°C with ^{125}I-GRP (0.5 nM) in the absence (●) and presence (○) of 10 μM bombesin. After centrifugation, the membranes were solubilized with 0.5% TDOC (A) or 0.5% DOC (B) as described in section 2 at a final protein concentration of 4 mg/ml. The supernatant (200 μl) obtained was analyzed by gel filtration as described in section 2. Typically, Blue Dextran 2000 eluted in fraction numbers 12 and 13 whereas free ^{125}I-GRP eluted between fractions 20–26. (C) Solubilization of ^{125}I-GRP-receptor complexes occurred when the ligand was prebound to the membrane receptor prior to detergent extraction. Swiss 3T3 membranes (1 mg) were incubated in the binding medium in the absence (△) or presence (○) of ^{125}I-GRP (0.5 nM), for 10 min at 37°C. The membranes were immediately solubilized without centrifugation in 0.5% TDOC for 30 min at 4°C. Following removal of non-extractable material by centrifugation, the supernatant of the non-prelabelled membranes (△) was incubated with 1 nM ^{125}I-GRP at 37°C for 1 h. The samples (200 μl) were then applied and eluted from Sephadex G-100 columns as described in section 2. In the presence of 10 μM bombesin, the profiles (omitted for clarity) were similar to the non-prelabelled membranes (△). (D) Dissociation of the ^{125}I-GRP-receptor complex at 4°C or 37°C. Swiss 3T3 membranes (1 mg) prelabelled with ^{125}I-GRP, were solubilized with 0.5% DOC (at 4 mg protein/ml) and applied to a Sephadex G-100 column as described in section 2. The fractions corresponding to the ^{125}I-GRP-receptor complex (12 and 13), were pooled (insert, ■). Half this pool was maintained at 4°C (○) and half incubated at 37°C for 1 h (●). Then, both samples were applied to identical Sephadex G-100 columns, and eluted as described in section 2.

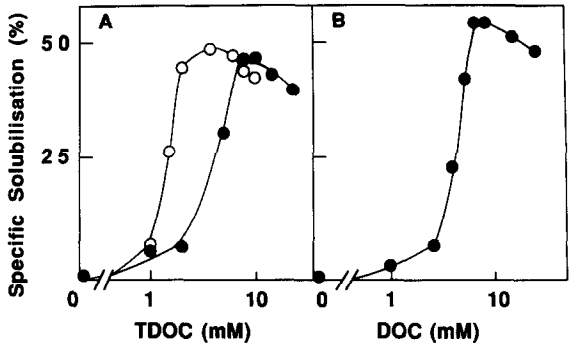

Fig. 2. (A) Extraction of ^{125}I-GRP complex as a function of detergent concentrations. Swiss 3T3 cell membranes (1 mg) were incubated for 10 min at 37°C with 0.5 nM GRP in binding medium in the absence or presence of 10 μM bombesin. The membranes were resuspended either at 5 mg protein/ml (closed circles) or at 1 mg protein/ml (open circles) and then solubilized as described in section 2 in the presence of different concentrations of either TDOC or DOC as indicated. The solubilized ^{125}I-GRP-receptor complex at each detergent concentration was determined on Sephadex G-100 columns and the percentage of specific solubilized complex expressed as described in section 2.

peak of specifically bound radioactivity was detected in the void volume of the G-100 column (results not shown). Thus, TDOC and DOC solubilized a high molecular weight component that has bound ^{125}I-GRP in a specific manner.

To determine whether the solubilized ^{125}I-GRP-receptor complex is dissociable, the radioactive peak that elutes in the void volume on gel filtration (Fig. 1D insert) was incubated either at 4°C or at 37°C for 60 min and then re-chromatographed at 4°C on a G-100 column. Fig. 1D shows that after incubation at 37°C, the radioactivity is markedly shifted from the void volume to the position of free ^{125}I-GRP, indicating dissociation of the ligand-receptor complex.

TDOC and DOC promoted solubilization of ^{125}I-GRP-receptor complexes in a dose-dependent manner (Fig. 2). Maximum solubilization by TDOC and DOC was achieved at 10 and 8 mM, respectively (0.4% w/v in both cases). The optimum detergent concentration was dependent on the detergent to protein ratios; a lower TDOC concentration (3 mM) was required for maximum solubilization in the presence of lower membrane protein (Fig. 2A). A variety of other detergents including Triton X-100, octylglucoside, dodecyl-β-D-maltoside, digitonin, cholate and CHAPS were much less effective than either TDOC or DOC in promoting ^{125}I-GRP-receptor solubilization as judged by both gel filtration and spun-column chromatography (Table I).

Previous studies using permeabilized 3T3 cells [21] or

Table I

Solubilization of ^{125}I-GRP-receptor complexes by various detergents

Detergent	Specific solubilization (%)	
	Spun column[b]	G-100[a]
Triton X-100	2 (1%)[d]	6 (1%)
Octylglucoside	3 (1%)	4 (1.5%)
n-Dodecyl-β-D-maltoside	4 (1%)	4 (0.5%)
Digitonin	12 (2%)	4 (1.5%)
CHAPS	15 (2%)	12 (1%)
Cholate	N.D.[c]	14 (1%)
DOC	52 (1%)	50 (1%)
TDOC	55 (2%)	48 (1%)

[a] The protein concentration during solubilization was 5 mg/ml
[b] Protein concentration during solubilization was 4 mg/ml
[c] Not determined
[d] Detergent concentrations are shown in brackets. CHAPS was also tested at 0.5% with similar results

Swiss 3T3 membranes were prelabelled with ^{125}I-GRP and solubilized with the detergents listed above as described in section 2. Specific solubilization of the ^{125}I-GRP-receptor complex was determined either by spun-column or Sephadex G-100 column chromatography as indicated

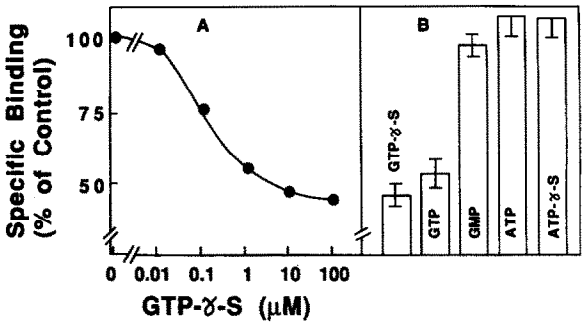

Fig. 3. GTPγS promotes dissociation of ^{125}I-GRP from ^{125}I-GRP-receptor complexes in a concentration-dependent (A) and -specific (B) manner. Swiss 3T3 membranes (2 × 1.0 mg) were labelled with ^{125}I-GRP (0.5 nM) in binding medium at 37°C for 10 min, solubilized with 0.5% DOC and chromatographed on two identical Sephadex G-100 columns as described in section 2. Fractions containing the solubilized ^{125}I-GRP receptor were then pooled. Aliquots (50–100 μl) were incubated either in the absence or presence of GTPγS at the indicated concentrations (A) or the specified nucleotides as indicated all at 100 μM (B). Following incubation of the reaction mixture at 37°C for 30 min the dissociated free ^{125}I-GRP was separated from the ^{125}I-GRP-receptor complex by spun-column chromatography as described in section 2. The results are expressed as the percentage of ^{125}I-GRP which remains bound to the receptor complex with respect to the control. The data represent the means ± SE; $n = 6$.

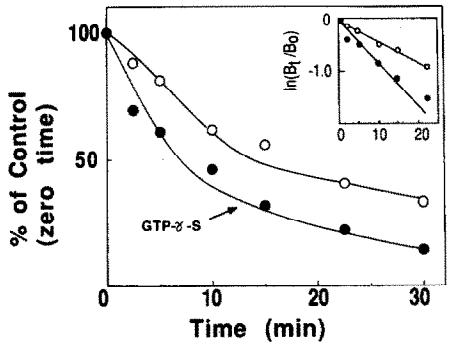

Fig. 4. Time course of the dissociation of ^{125}I-GRP from the solubilized ^{125}I-GRP-receptor complex in the absence and presence of GTPγS. Swiss 3T3 membranes (2 × 1.0 mg) were prelabelled with ^{125}I-GRP (0.5 nM) solubilized with 0.5% DOC and chromatographed on two identical Sephadex G-100 columns as described in section 2. Aliquots (50 μl) of the pooled fractions containing the ^{125}I-GRP-receptor complex were incubated in the absence (○) or in the presence (●) of 100 μM GTPγS for the indicated times, at 37°C. Free ^{125}I-GRP was separated from bound peptide by spun-column chromatography as described in section 2. The results represent the average of two independent experiments. (Inset) Semi-logarithmic plot of the data. Time was plotted on the abscissa and $\ln(B_t/B_o)$ was plotted on the ordinate (where B_t is the bound ^{125}I-GRP at the indicated times and B_o ^{125}I-GRP bound at time zero).

membrane preparations of these cells [13] suggested that the bombesin receptor is coupled to a G protein. Several hormone receptors known to be functionally coupled to G proteins remain physically associated with them after detergent solubilization [16–20]. To determine whether the solubilized ^{125}I-GRP-receptor complex is functionally coupled to a G protein(s) we tested whether the ligand-receptor complex isolated by gel filtration retains the ability to be regulated by guanine nucleotides. Swiss 3T3 membranes were incubated with ^{125}I-GRP and then solubilized with DOC. The detergent-solubilized extract was chromatographed on a G-100 column and the ligand-receptor complex eluting in the void volume was collected. The pooled fractions were incubated in the absence or in the presence of increasing concentrations of the non-hydrolyzable GTP analogue, GTPγS for 30 min at 37°C. Then, bound ^{125}I-GRP was separated from dissociated ligand by spun-column chromatography. As shown in Fig. 3A, GTPγS caused a dose-dependent decrease in the level of ^{125}I-GRP-receptor complex. Half-maximal and maximal effects were achieved at 0.12 and 10 μM, respectively. The specificity of this effect is shown in Fig. 3B. Addition of GMP, ATP or ATPγS at 100 μM did not have any detectable effect on the stability of the solubilized ^{125}I-GRP-receptor complex whereas either GTPγS or GTP caused a 50% reduction in the level of bound ^{125}I-GRP in parallel samples. Similar results were obtained when the separation of bound from unbound ^{125}I-GRP was carried out by rechromatography on Sephadex G-100 columns (as in Fig. 1D) instead of by spun-column chromatography (results not shown).

We next examined the time-course of dissociation of the solubilized ^{125}I-GRP-receptor complex in the absence or presence of GTPγS. As shown in Fig. 4, addition of GTPγS caused a marked increase in the rate of dissociation of the solubilized complex. The rate constant of dissociation increased from 0.04 min^{-1} to 0.086 min^{-1} in the presence of GTPγS. The guanine nucleotide sensitivity shown in Figs 3 and 4 strongly suggests that the solubilized ^{125}I-GRP-receptor complex is physically associated to a G protein.

In conclusion, the present study demonstrates for the first time the successful solubilization of ^{125}I-GRP-receptor complexes from Swiss 3T3 cell membranes and provides functional evidence for a bombesin/GRP receptor-G protein interaction. We show that GTPγS enhanced the rate of dissociation of ^{125}I-GRP from the solubilized ligand-receptor complex in a dose-dependent and -specific manner. These findings, together with previous studies with membrane preparations [13] and permeabilized cells [21], indicate that the mitogenic bombesin receptor elicits intracellular responses via a G protein signal transduction pathway. Finally, the solubilization of the bombesin receptor in an active form may prove an important step for attempting its purification and reconstitution into phospholipid vesicles.

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