Solubilization of the Receptor for the Neuropeptide Gastrin-Releasing Peptide (Bombesin) with Functional Ligand Binding Properties

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ABSTRACT: The receptor for the neuropeptide gastrin-releasing peptide, the mammalian homologue of bombesin, was solubilized from rat brain and Swiss 3T3 cells by using the zwitterionic detergent 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid (CHAPS) and the cholesteryl hemisuccinate ester (CHS). Only the combination of the detergent CHAPS and the cholesteryl ester CHS in a glycerol-containing buffer satisfactorily preserved the binding activity upon solubilization. Specific binding activity was only solubilized from cell lines and tissue preparations known to express the GRP receptor. The dissociation constant (K_d) for the receptor solubilized from rat brain and Swiss 3T3 cells was 0.6 nM, similar to the value of 0.8 nM calculated for the membrane-bound receptor. Binding was saturable and reached equilibrium after approximately 2 h at 4 °C. The identity of the solubilized receptor with the membrane-bound one was further confirmed by the concordance of the relative binding affinities of various established bombesin analogues.

The bombesin (Anastasi et al., 1971) and gastrin-releasing peptide (GRP)[1] (McDonald et al., 1979) family of neuropeptides is known for its wide array of both central nervous system and peripheral effects (Sunday et al., 1988). In particular, it causes increased grooming (Gmerek & Cowan, 1982), decreased food intake (Stuckey et al., 1982), and altered hormone secretion (Tache & Gunion, 1985) following injection into the rat central nervous system. These neuropeptides are also known for their mitogenic activity on selected tissues and cells, including human bronchial epithelial cells (Willey et al., 1984), small cell lung cancer cells (Carney et al., 1987; Cuttitta et al., 1985), and murine Swiss 3T3 fibroblasts (Rozengurt & Sinnett-Smith, 1983; Zachary et al., 1987). Key for elucidating the mechanism of such a pleiotropism of effects is the molecular characterization of the GRP membrane receptor molecule(s). Receptors for GRP, which recognize the conserved C-terminus of bombesin and GRP, are present throughout the central nervous system (Zarbin et al., 1985), the gastrointestinal tract (Jensen et al., 1978), and other target organs. They have been identified and characterized mostly by receptor autoradiography and radioreceptor assay.

By use of cultured cell lines, a number of membrane proteins were reported to be specifically labeled by chemical cross-linking techniques using radiolabeled ligand. These putative receptors exhibit different molecular weights in different target cell types: from 65K to 85K on mouse 3T3 fibroblast cells (Kris et al., 1987; Zachary & Rozengurt, 1987), 75K on the human glioma cell line GM-340 (Kris et al., 1987), and about 100K in the rat pituitary cell line GH_4C_1 (Fischer & Schonbrunn, 1988). At least for the 3T3 cell lines, the difference in size of the receptor was ascribed to differential glycosylation of a protein core of approximately 45K (Kris et al., 1987). An unequivocal characterization of the receptor molecule must await the biochemical purification of its binding activity to homogeneity or the expression cloning of the GRP receptor. The first step toward protein purification is the successful solubilization of the receptor in an active form.

The design of a protocol for solubilization of a membrane receptor protein still appears to be rather empirical. A number of nonionic detergents are available for trial featuring both low denaturing potential and a high critical micellar concentration (cmc), allowing for easier detergent removal (Hjelmeland & Chrambach, 1984). Often the addition of other classes of compounds, and in particular, lipids, helped in stabilizing the molecule (Levitzki, 1985).

Here we solubilized the GRP receptor employing a combination of the detergent 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid (CHAPS) and the ester cholesteryl hemisuccinate (CHS) in a glycerol-containing buffer, previously employed for the solubilization of the neurotensin receptor (Mazella et al., 1988) and the γ-aminobutyric acid/benzodiazepine receptor (Bristow & Martin, 1987). A soluble receptor assay was developed employing poly(ethylenimine)-precoated glass fiber filters (Bruns et al., 1983). Optimal conditions for binding were devised, including ligand and receptor stability and medium composition. Mo-

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[1] Abbreviations: CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; CHS, cholesteryl hemisuccinate; cmc, critical micellar concentration; DMEM, Dulbecco's modified Eagle's medium; EDTA, ethylenediaminetetraacetic acid; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; GRP, gastrin-releasing peptide; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PBS, phosphate-buffered saline; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate.

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novalent cations inhibited binding to the receptor, whereas low concentrations of divalent cations were required for maximum binding. The identity of the solubilized GRP receptor was then established by its specific extraction only from receptor-bearing cell lines and tissues, and by the binding parameters determined for a number of bombesin/GRP analogues.

Materials and Methods

Materials. [Tyr^4]bombesin and the other peptide analogues were purchased from Peninsula Laboratories. ^{125}I-GRP was purchased from Amersham Corp. Other products were from the following sources: CHAPS (Calbiochem); CHS, PMSF, leupeptin, pepstatin, aprotinin, and bacitracin (Sigma). All other chemicals were at least of reagent grade, and solvents used were HPLC-grade. Tissue culture products were purchased from Gibco. Protein concentrations were determined with the protein assay reagent BCA (Pierce).

Cells. Swiss and NIH 3T3 cell lines were grown in DMEM medium with 10% calf serum. A431 cells were grown in DMEM medium with 10% fetal calf serum.

Radioiodination of [Tyr^4]bombesin and HPLC Analysis of Radiolabeled Peptide. [Tyr^4]bombesin (1.5 μg) (Peninsula) was labeled with 2 mCi of ^{125}INa (Amersham) by incubating with 0.9 μg of chloramine T (Sigma) in 30 μL of 0.1 M phosphate buffered to pH = 7.4 for 1 min at room temperature. The reaction was stopped by 1.4 μg of sodium metabisulfite (Sigma), and the radiolabeled ligand was separated from free $^{125}I_2$ by chromatography on a Sephadex LH-20 (Pharmacia-LKB) column (15 cm × 20 cm) preequilibrated and run in methanol/acetic acid/H_2O (10:2:1). Fractions containing the organic peak of radioactivity were then pooled and dried down to approximately 300 μL in a Savant vacuum desiccator. Dithiothreitol (Sigma) was then added to a final concentration of 1 M and a dilution factor of 4, and the mixture was incubated for 2 h at 80 °C to effect the reduction of the C-terminal methionine sulfoxide generated by the oxidizing conditions of the iodination procedure.

Excess dithiothreitol and the remaining free iodine were then removed by filtration through a C-18 Sep-pak cartridge (Waters) which was then washed with H_2O. The retained material was then eluted with 35% acetonitrile (Burdick-Jackson) in H_2O supplemented with 0.1% trifluoroacetic acid (Applied Biosystems), dried down to 10–20 μL in a Savant vacuum desiccator, diluted at least 25-fold with H_2O containing 0.25% w/v bovine serum albumin (fraction V, protease-free, Calbiochem), 20 mM dithiothreitol, and 100 μg/mL aprotinin, and dispensed in 25-μL aliquots in siliconized tubes to be stored frozen at −20 °C. The radiolabeled peptide was stable for at least 3 weeks. HPLC analysis of the radiolabeled material was performed on a C-18 analytical column (Vydac) using a Hewlett-Packard 1090 liquid chromatograph. The column was resolved with a gradient of acetonitrile (from 10% to 30%, 0.5% increase per minute) in H_2O containing 0.1% trifluoroacetic acid and the radioactivity in the eluant monitored with an LKB 1208 Betacord radioactivity monitor. The radiolabeled material eluted from the Sep-pak cartridge contained virtually no free $^{125}I_2$ and contained mostly monoiodo ^{125}I-[Tyr^4]bombesin. The specific activity of the tracer was approximately 1500 ci/mmol and was determined by competition experiments with different concentrations of unlabeled [Tyr^4]bombesin for binding to Swiss 3T3 membranes.

Preparation of Membrane Fraction from Swiss 3T3 Cells. Confluent cultures of Swiss 3T3 cells were rinsed three times with phosphate-buffered saline (PBS) and scraped with a rubber policeman in ice-cold PBS containing 1 mM PMSF on ice. Cells were then pelleted, washed once with excess ice-cold lysis buffer (50 mM Tris, pH = 8.0, 1 mM EGTA, 5 mM $MgCl_2$, and the following protease inhibitors: leupeptin at 50 μg/mL, pepstatin at 5 μg/mL, aprotinin at 10 μg/mL, bacitracin at 200 μg/mL, and 1 mM PMSF), and then homogenized in a large volume of the same buffer with a Dounce homogenizer and a glass type B pestle on ice (30–40 strokes). Nuclei and other debris were then pelleted by a low-speed centrifugation (800*g* for 5 min at 4 °C); the supernatant was set aside on ice, and the pellet was reextracted with the same volume of lysis buffer (20 strokes). After another low-speed centrifugation both supernatants were pooled and centrifuged at 20000*g* for 30 min at 4 °C. The crude membrane pellet was resuspended in HEMI buffer with the aid of a Dounce homogenizer. The protein content was measured, glycerol was added to a final concentration of 30%, and the suspension was aliquoted, quickly frozen in methanol–dry ice, and stored at −80 °C.

Preparation of Membrane Fractions from Rat Brains. Young male Sprague-Dawley rats were euthanized by decapitation after ether anesthesia, and the brains were quickly dissected out, rinsed in ice-cold TI buffer (10 mM Tris, pH = 7.4, and the protease inhibitors listed above), and homogenized in a large volume of ice-cold TI buffer with a polytron (20 s in an ice bath). The homogenate was then centrifuged at 150000*g* for 20 min at 4 °C, and the pellet was resuspended in an excess volume of TI supplemented with 0.15 M NaCl, allowed to sit on ice for 1 h, and again centrifuged as above. The pellet was resuspended in an excess volume of TI and again centrifuged as above and the new pellet was finally resuspended in HEMGI buffer. Protein content was meausred, and membranes were repelleted again as above and immediately extracted as described below.

Binding Experiments with Membrane Preparations. Approximately 150 μg of Swiss 3T3 crude membrane preparation from a stock stored frozen at −80 °C in the presence of 30% glycerol as suspended in a final volume of 100 μL with HEMI buffer (20 mM HEPES–KOH, pH = 6.8, 1 mM EGTA, 5 mM $MgCl_2$, and the following protease inhibitors: leupeptin at 50 μg/mL, aprotinin at 10 μg/mL, pepstatin at 5 μg/mL, bacitracin at 200 μg/mL, and 1 mM PMSF) and incubated with 0.5 nM ^{125}I-[Tyr^4]bombesin for different times. At the end of the incubation samples were put in an ice bath and filtered under vacuum suction through Whatman GF/C glass fiber filters, pretreated with 0.3% poly(ethylenimine) (Eastman Kodak) in H_2O for at least 2 h. Filters were washed twice with 8 mL of ice-cold 20 mM HEPES–KOH, pH = 6.8, and counted in an LKB γ-counter with a counting efficiency of 60%. Nonspecific binding was determined by parallel incubation of the membranes in the presence of a 1000-fold excess of unlabeled [Tyr^4]bombesin and was approximately 10% of total binding. Specific binding was calculated by subtracting nonspecific binding from total bound.

For equilibrium binding experiments, saturation curves were obtained by incubating the membranes for 30 min at 37 °C with increasing concentrations of ^{125}I-[Tyr^4]bombesin. Data were then analyzed according to the method of Scatchard with a computer program written by Dr. Stephen Felder (Rorer Biotechnology). Uncertainties of equilibrium binding parameters were calculated from the curvature of the error matrix of the least-squares fitting procedure.

Cross-linking experiments on Swiss 3T3 membrane preparations were performed by using ^{125}I-GRP (Amersham) and the cross-linking agent disuccinimidyl suberate (Pierce) as previously described (Kris et al., 1987).

Solubilization Protocol and Soluble Receptor Assay. Membrane preparations were solubilized at a protein concentration of 15 mg/mL with 0.75% (w/v) CHAPS and 0.15% (w/v) CHS [diluted from a 1.25% CHAPS/0.25% CHS (w/v) stock in 20 mM HEPES, pH = 6.8, and 10% glycerol (v/v), stored frozen at −20 °C] in HEMGI buffer [20 mM HEPES–KOH, pH = 6.8, 1 mM EGTA, 5 mM $MgCl_2$, 10% glycerol (v/v), and the following protease inhibitors: leupeptin at 50 μg/mL, aprotinin at 10 μg/mL, pepstatin at 5 μg/mL, bacitracin at 200 mg/mL, and 1 mM PMSF] by gentle stirring for 1 h at 4 °C. The addition of 10% glycerol to the solubilization buffer was found to markedly improve the stability of the solubilized receptor. The solubilized receptor in the crude extract was more stable at 4 °C than at higher temperatures, with a half-life at 4 °C of approximately 60 h, and could be stored frozen at −80 °C for several weeks without appreciable loss of activity. Extracts were then cleared by ultracentrifugation at 100000*g* for 1 h and either used immediately or quickly frozen at −80 °C. For binding experiments, extracts were diluted to 0.1% or 0.2% CHAPS with HEMGI buffer and incubated with 0.5 nM ^{125}I-[Tyr^4]bombesin for different times at 4 °C. Saturation curves were obtained by incubating the extracts for 3 h at 4 °C. At the end of the incubation time, samples were filtered under vacuum suction through Whatman GF/F glass fiber filters pretreated with 0.3% poly(ethylenimine) (v/v) (Eastman Kodak) in H_2O for at least 2 h. Filters were then processed and binding data analyzed as described above for binding experiments with membrane preparations. Nonspecific binding, determined by incubation of parallel samples in the presence of an excess (1 μM) of unlabeled [Tyr^4]bombesin, was 10%–30% of the total bound.

For dissociation kinetics experiments, extracts were preincubated for 3 h at 4 °C with 0.1 nM ^{125}I-[Tyr^4]bombesin, diluted 15-fold with HEMGI or other buffers supplemented with 100 nM unlabeled bombesin, and further incubated for different times. At the end of the second incubation, the remaining specific binding was determined as above.

The fraction of solubilized receptors detected by the assay was estimated from the fraction of radioactivity retained on the filter from a dialyzed extract of Swiss 3T3 membrane prelabeled with ^{125}I-GRP and a chemical cross-linker. No degradation of the radiolabeled ligand occurred during incubation with the extract, as assessed by reverse-phase HPLC.

Gel Filtration of Soluble Receptor. Swiss 3T3 extract (0.5 mL) at 7 mg/mL in 0.75% CHAPS/0.15% CHS was loaded onto a 30-mL bed of Sephacryl S-300 superfine (Pharmacia) packed in a 1.5 cm × 20 cm column and preequilibrated with 0.1% CHAPS/0.02% CHS in HEMG buffer at 4 °C. The column was eluted with the same buffer, and fractions of 1 mL were collected and analyzed for the presence of soluble binding activity as described above. The column was calibrated with parallel runs of the following gel filtration standards (Bio-Rad): blue dextran, thyroglobulin catalase, bovine serum albumin, ovalbumin, myoglobin, and vitamin B-12.

Results

Binding Parameters of ^{125}I-[Tyr^4]bombesin to Swiss 3T3 Membranes. Suspensions of crude membrane pellets prepared from Swiss 3T3 cells were assayed for specific binding of the radiolabeled bombesin analogue ^{125}I-[Tyr^4]bombesin. The specific binding, which amounted to 90% of the total, increased with time, reaching a plateau after 15 min at 37 °C and approximately 2 h at 4 °C (Figure 1).

Analysis of the binding experiments according to the method of Scatchard showed the presence of a single class of high-affinity binding sites with a dissociation constant of 0.83 nM at 4 °C and a saturation density of 240 fmol of receptor/mg of protein (data not shown). The calculated K_d for the membrane-bound receptor of 3T3 Swiss cells was in close agreement with the values previously reported for intact cells (Zachary & Rozengurt, 1985; Kris et al., 1987).

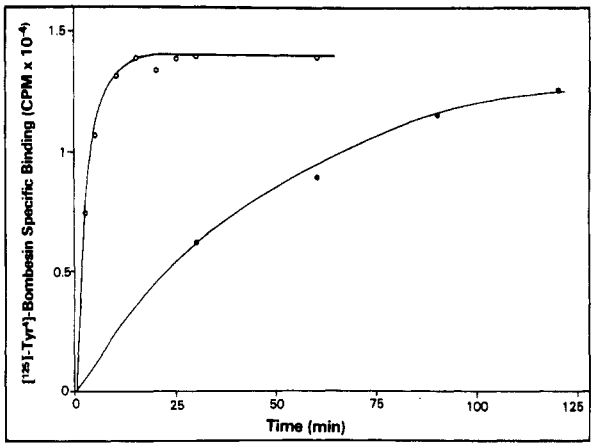

FIGURE 1: Association kinetics of ^{125}I-[Tyr^4]bombesin with Swiss 3T3 membranes at 37 and 4 °C. Samples (150 μg) from a crude membrane preparation of Swiss 3T3 cells were incubated with approximately 0.5 nM of ^{125}I-[Tyr^4]bombesin for the indicated time at 37 °C (open circles) or at 4 °C (closed circles). Specific binding was determined as described under Materials and Methods. Nonspecific binding determined by incubating the membranes in the presence of a thousandfold excess of unlabeled [Tyr^4]bombesin was less than 10% of the total. The concentration of free ligand did not vary more than 10% during the course of the experiment. Values shown are the means of triplicate determinations from a representative experiment.

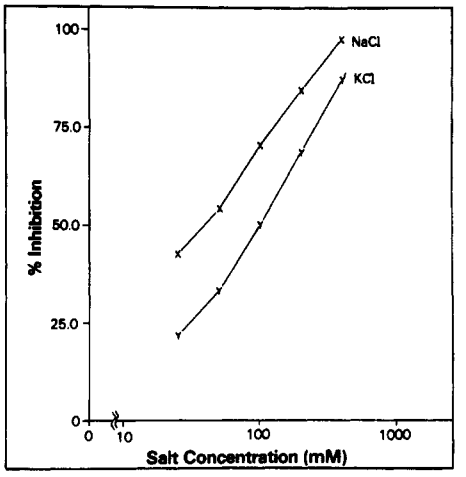

FIGURE 2: Inhibition of ^{125}I-[Tyr^4]bombesin specific binding to Swiss 3T3 membranes by monovalent cations. Samples (150 μg) from a crude membrane preparation of Swiss 3T3 cells were incubated for 30 min at 37 °C with 0.5 nM ^{125}I-[Tyr^4]bombesin and various concentrations of NaCl (X) or KCl (Y). Specific binding was determined as described under Materials and Methods. Data are expressed as percentage of inhibition of ^{125}I-[Tyr^4]bombesin specific binding measured in the absence of added cation. Each point is the mean of triplicate determinations.

The effect of various salts on binding was evaluated. NaCl inhibited the binding in a concentration-dependent manner with an IC_{50} of 46 mM (Figure 2). KCl exerted a similar but less potent effect, with an IC_{50} of 166 mM (Figure 2), suggesting that the cation is the main source of the inhibition. The inhibition of binding by NaCl was reversible (data not shown). In contrast, a low concentration of divalent cations was required for optimal binding. Higher concentrations of divalent cations were again inhibitory, with $CaCl_2$ more effective (IC_{50} = 36 mM) than $MgCl_2$ (IC_{50} = 67 mM). The

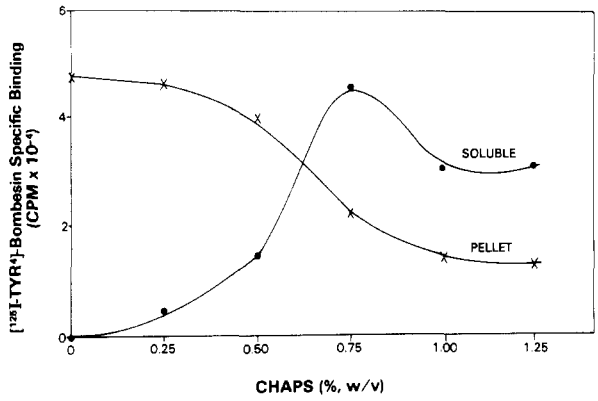

FIGURE 3: Solubilization of bombesin/GRP receptor from Swiss 3T3 membranes. Samples (50 μg) from a crude membrane preparation of Swiss 3T3 cells were extracted at 5 mg/mL with the indicated concentration of CHAPS plus one-fifth of that concentration of CHS for 1 h at 4 °C. After ultracentrifugation at 100000*g* for 1 h ^{125}I-[Tyr^4]bombesin specific binding was analyzed in the pellet (×) and in the soluble fraction (closed circles) after bringing all samples to the same concentration of detergent (0.1% w/v), as described under Materials and Methods. Each point is the mean of triplicate determinations.

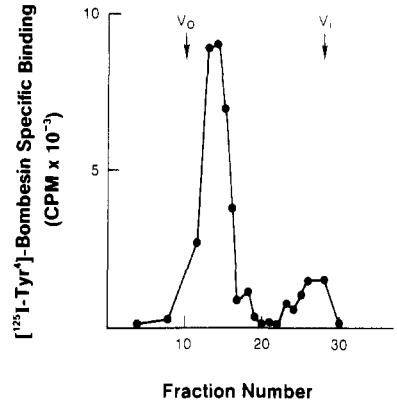

FIGURE 4: Gel filtration chromatography of CHAPS-solubilized bombesin/GRP receptor. Swiss 3T3 extract (0.5 mL) was chromatographed on a Sephacryl S-300 column preequilibrated in HEMGI buffer containing 0.1% CHAPS and 0.02% CHS at 4 °C. Fractions (1 mL) were collected and analyzed for binding activity was described under Materials and Methods. The void volume (V_0 = elution volume of blue dextran, average M_r = 2000000) and included volume (V_i = elution volume of vitamin B_{12}, M_r = 1350) are indicated by the arrows. The elution volumes of the markers were as follows: thyroglobulin (M_r = 670000), 12 mL; catalase (M_r = 200000), 13 mL; bovine serum albumin (M_r = 66000), 17 mL; ovalbumin (M_r = 44000), 21 mL; myoglobin (M_r = 17000), 23 mL.

pH dependency of the binding was also investigated, and a pH optimum was found between 6.4 and 6.8 (data not shown).

Solubilization of GRP Receptor. A filtration assay through poly(ethylenimine)-precoated glass fiber GF/F filters effected the best separation between free and bound ligand, with a detection of up to 85% of the solubilized receptor and a background retention of approximately 1% of the added CPM of ^{125}I-[Tyr^4]bombesin. When the receptor was solubilized by CHAPS in the presence of CHS, the specific binding was between 65% and 80% of the total bound and the sensitivity of the assay was of about 6 fmol of receptor per sample (data not shown). A dose–response curve for the amount of solubilized protein showed the expected linear relationship of protein vs assayed activity (data not shown). No saturation of the assay was observed for loading up to 1.5 mg of protein per filter.

When crude membrane pellets from Swiss 3T3 cells were extracted with increasing concentration of CHAPS in the presence of CHS, an increasing amount of receptor was recovered in the soluble fraction. However, when the concentration of CHAPS was raised above 0.75% (w/v), a progressive inactivation of the solubilized receptor was noted (Figure 3). Optimal solubilization was hence obtained with 0.75% (w/v) CHAPS and was virtually complete by 1 h of extraction at 4 °C. An additional extraction of the pellet did not improve the yield. Approximately 90% of the binding sites assayed in the initial membrane preparation were recovered in the soluble extract.

The solubilized binding activity was recovered in the supernatant fraction after centrifugation of the extract at 100000*g* for 1 h at 4 °C. To further prove its soluble nature, 0.5 mL of Swiss 3T3 cell extract in 0.75% CHAPS and 0.15% CHS were cleared by ultracentrifugation and loaded on a 30-mL Sephacryl Superfine S-300 column preequilibrated in solubilization buffer with 0.1% detergent at 4 °C. Fractions from the column were then assayed for the presence of specific bombesin binding activity. This was found to partition in the column between the void and the included volume with an elution volume corresponding to an apparent molecular size of approximately 200000 daltons (Figure 4). When a sample of membranes subjected to chemical cross-linking after equilibrium binding with radiolabeled GRP was solubilized with CHAPS and loaded onto the same gel filtration column,

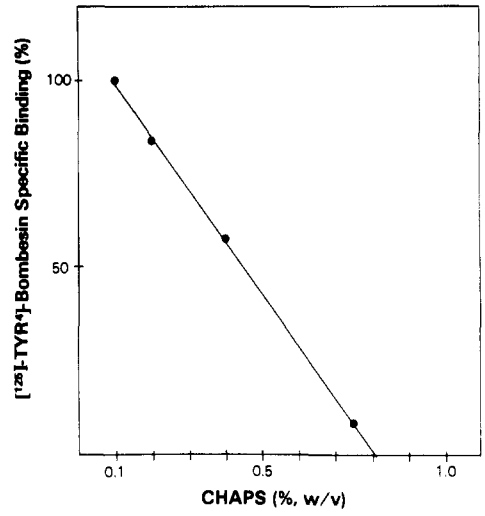

FIGURE 5: Effect of detergent concentration on binding activity of soluble bombesin/GRP receptor. Samples of crude membrane preparations of Swiss 3T3 cells were extracted with 0.75% CHAPS/0.15% CHS in HEMGI buffer, and the soluble fractions were diluted to the indicated detergent concentration in the same final volume of HEMGI buffer and assayed for bombesin binding activity as described under Materials and Methods. Values shown are the means of triplicate determinations.

the prominently labeled 75-kDa protein (as identified by SDS–polyacrylamide gel electrophoresis) eluted in the same volume of the assayed soluble binding activity (data not shown).

Optimal binding was obtained when the detergent concentration was diluted to 0.1%, (Figure 5). The dependence of receptor solubilization on the concentration of protein in the extraction buffer was also investigated, and a concentration of 15 mg/mL protein was found to give the optimal yield of solubilized receptor per milligram of extracted protein (data not shown).

Cell and Tissue Specificity. High-affinity specific binding of solubilized material was only detectable from cell lines previously shown to express the GRP receptor. The NIH 3T3-3 mouse fibroblast cell line and the human epidermoid carcinoma A431 cell line, previously shown not to express the

Table I: Specificity of the Soluble Receptor Assay for Bombesin/GRP Receptor[a]

source of extract	^{125}I-[Tyr^4]bombesin specific binding (fmol/mg)
Swiss 3T3 cells	65 ± 4
NIH 3T3-3 cells	0
A431 cells	0
rat brain membranes	11.6 ± 2.6
bovine brain membranes	0.37 ± 0.26

[a] Different cell lines or brain membrane preparations were extracted with 0.75% CHAPS and 0.15% CHS in HEMGI buffer, and the soluble fractions were assayed for the presence of specific bombesin binding activity with 0.5 nM ^{125}I[Tyr^4]bombesin as described under Materials and Methods. The values shown are the means and standard deviations from three different determinations.

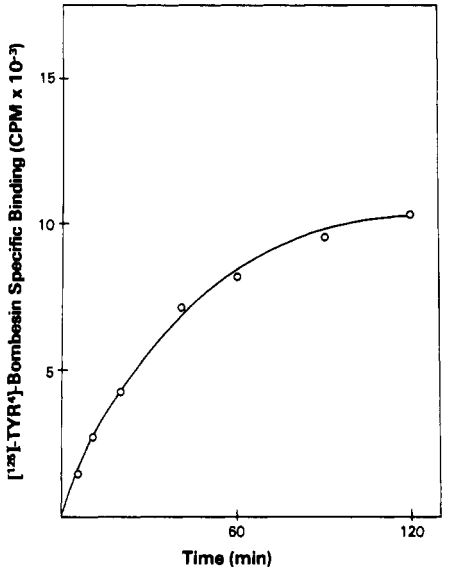

FIGURE 6: Association kinetics of ^{125}I-[Tyr^4]bombesin to soluble receptor from Swiss 3T3 cells at 4 °C. Proteins (40 μg) solubilized from Swiss 3T3 membrane preparation with 0.75% CHAPS/0.15% CHS in HEMGI buffer were diluted to 0.1% detergent with HEMGI buffer and incubated with 0.5 nM ^{125}I-[Tyr^4]bombesin for the indicated time at 4 °C. Specific binding was determined as described under Materials and Methods. Nonspecific binding was determined by incubating the extracts in the presence of a thousandfold excess of unlabeled [Tyr^4]bombesin and was between 10% and 20% of the total counts. The concentration of free ligand did not vary more than 10% during the course of the experiment. Values shown are the means of triplicate determinations from a typical experiment.

GRP receptor (Kris et al., 1987), scored negative also in the soluble receptor assay (Table I). A functional receptor could be solubilized from tissue preparations where its presence had been previously demonstrated (Zarbin et al., 1985), such as rat or bovine brain membrane preparations (Table I).

Kinetics and Equilibrium Binding Parameters. The kinetics of ^{125}I-[Tyr^4]bombesin binding to the receptor solubilized from crude membrane pellets of Swiss 3T3 cells was studied at 4 °C and in a manner similar to the one described above for the membrane-bound receptor. The ligand was found to associate with the solubilized receptor, reaching equilibrium after approximately 2 h (Figure 6).

A Scatchard analysis of the binding of ^{125}I-[Tyr^4]bombesin to the solubilized receptor showed the presence of a single class of high-affinity binding sites with a dissociation constant of 0.62 nM at 4 °C and a saturation density of 260 fmol of receptor/mg of protein (data not shown). A similar analysis with the receptor solubilized from rat brain membranes gave a single class of high-affinity binding sites with a dissociation constant of 1.1 nM at 4 °C and a saturation density of 90 fmol/mg of protein (data not shown). The affinity of the receptor for its ligand is not significantly affected by the solubilization protocol.

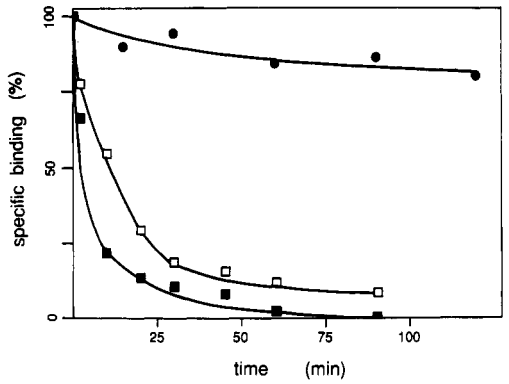

FIGURE 7: Dissociation kinetics of ^{125}I-[Tyr^4]bombesin from soluble Swiss 3T3 receptor at 4 °C. Effect of ionic conditions. Proteins (50 μg) solubilized from Swiss 3T3 cells were incubated with 0.1 nM ^{125}I-[Tyr^4]bombesin for 3 h at 4 °C. Samples were then diluted 15-fold with the following buffers containign 100 nM unlabeled [Tyr^4]bombesin and further incubated at 4 °C for the indicated time: (●) control HEMI buffer; (□) 10 mM EDTA in HEMI buffer without $MgCl_2$; (■) 500 mM NaCl in HEMI buffer. At the end of the second incubation, the remaining specific binding was determined as described under Materials and Methods. Data are plotted as percentage of the specific binding measured for the control sample at the start of the second incubation and present the means of triplicate determinations from a representative experiment.

The dissociation of bound ^{125}I-[Tyr^4]bombesin was investigated. No specific binding to the soluble receptor was observed in the presence of 0.5 M NaCl (data not shown). The addition of this same concentration of salt after equilibrium binding of the radiolabeled ligand promoted its dissociation with a half-time of approximately 4 min at 4 °C. Chelation of the Mg^{2+} ions also prompted the dissociation of the bound ligand with a half-time of approximately 12 min at 4 °C. On the other hand, a very slow dissociation of bound ligand was observed on adding an excess of unlabeled bombesin at 4 °C (Figure 7). The effect of these ionic changes on the binding activity of the solubilized receptor was completely reversible, as shown by the full recovery of both total binding activity and the proper dissociation constant after dialysis of the treated sample (data not shown). The pH dependencey of the solubilized binding activity was also investigated, and a pH optimum was found between 6.8 and 7.0 (data not shown).

Comparison of the Potencies of Bombesin Analogues To Inhibit the Binding of Bombesin to either Membrane-Bound or Solubilized Receptor. To further evaluate the validity of the solubilization protocol, a panel of bombesin analogues were tested for their ability to displace ^{125}I-[Tyr^4]bombesin from membrane-bound and solubilized receptor. The agonist peptides tested were [Tyr^4]bombesin, GRP, the fourteen amino acid C-terminal moiety of GRP (GRP 14–27) neuromedin B, the ten amino acid C-terminal moiety of GRP (GRP 10), and the seven amino acid C-terminal bombesin septapeptide (bombesin 8–14). The antagonist peptide [D-Arg^1,D-Pro^2,D-Trp^{7,9},Leu^{11}]substance P (Jensen et al., 1984) and the inactive sixteen amino acid N-terminal moiety of GRP (GRP 1–16) were also tested. As shown in Figure 8, there appears to be a satisfactory degree of concordance between the two sets of displacement curves measured for the membrane-bound and the solubilized receptor. The relative potencies of these peptides were similar to those previously reported for the GRP receptor assayed in whole cells (Zachary & Rozengurt, 1985; Swope & Schonbrunn, 1987). GRP and [Tyr^4]bombesin were equipotent for both forms of the receptor, and as expected,

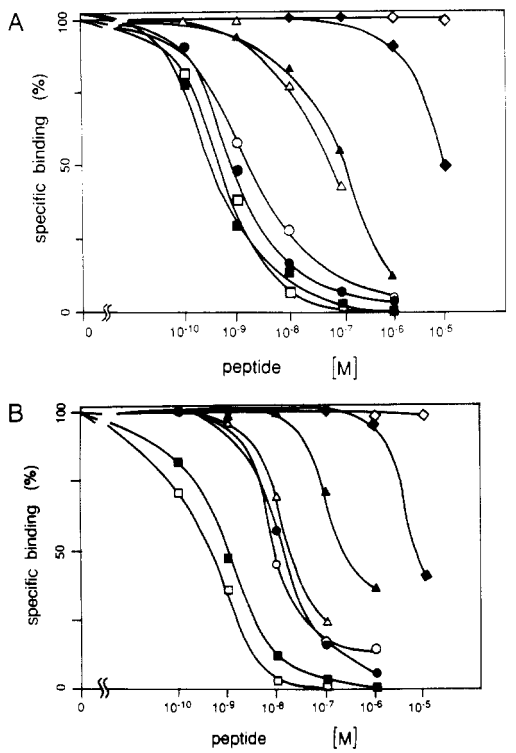

FIGURE 8: Inhibition of the specific binding of ^{125}I-[Tyr^4]bombesin to the membrane-bound and the soluble receptor from Swiss 3T3 cells by a spectrum of bombesin analogues. Sample containing either 100 μg of a crude membrane preparation of Swiss 3T3 cells (A) or 40 μg of proteins solubilized from it with CHAPS and CHS (B) were incubated to equilibrium binding (30 min at 37 °C for membranes and 3 h at 4 °C for extracts) with 0.5 nM ^{125}I-[Tyr^4]bombesin and the indicated concentration of analogue. Specific binding was then determined as described under Materials and Methods. Data are plotted as percentage of the specific binding measured in the absence of any added competitor and represent the means of triplicate determinations. The peptides tested were the following: (□) GRP; (■) [tyr^4]bombesin; (●) GRP 14–27; (○) GRP 10; (▲) neuromedin B; (♦) [D-Arg^1,D-Pro^2,D-Trp^{7,9},Leu^{11}]substance P; and (◇) GRP 1–16.

the concentration of them required to produce half-maximal inhibition of binding (IC_{50} around 0.5 nM) was the same as that of ^{125}I-[Tyr^4]bombesin used in the assay. GRP 14–27 and GRP 10 were slightly less potent for the membrane-bound form (IC_{50} around 1 nM) and significantly weaker for the soluble form (IC_{50} around 10 nM). Bombesin 8–14 and neuromedin B scored with a potency between 1 and 2 orders of magnitude lower than that of bombesin for both forms of the receptor (IC_{50} of approximately 60 nM and 150 nM for the membrane-bound form and 20 nM and 200 nM for the soluble form, respectively). Furthermore, 50% inhibition of ^{125}I-[Tyr^4]bombesin binding was observed both for the membrane-bound and the solubilized receptor with approximately 10 μM of the bombesin receptor antagonist [D-Arg^1,D-Pro^2,D-Trp^{7,9},Leu^{11}]substance P while no inhibition was exerted on either form by a similar concentration of the inactive N-terminal fragment of GRP.

Discussion

In this paper we show that the receptor for bombesin/GRP can be successfully solubilized by the combination of the zwitterionic detergent CHAPS and the cholesteryl ester CHS. While the receptor molecule was readily solubilized by a number of nonionic detergents, specific binding activity could only be recovered with the detergent CHAPS and CHS. This suggests a requirement of the bombesin/GRP receptor for a particular lipid milieu which the combination of CHAPS and CHS probably simulates. The need for lipids as stabilizers of receptor binding activity in the soluble state has been extensively reported, especially for receptors thought to couple with G proteins for signal transduction (Levitzki, 1985). In most cases, crude preparations of lipids such as soybean or egg lecithin performed best, but the requirement for specific classes of lipids was also evaluated. Cholesterol or cholesteryl hemisuccinate were found to improve the reconstitution efficiency of the nicotinic acetylcholine receptor into lipid vesicles (Levitzki, 1985). A specific stabilization of the soluble receptor by the ester cholesteryl hemisuccinate was reported for the neurotensin receptor (Mazella et al., 1988) and the γ-aminobutyric acid/benzodiazepine receptor (Bristow & Martin, 1987). Both receptors were solubilized also by using the detergent CHAPS.

The disrupting action of the detergent on the bombesin/GRP receptor, though, was still noticeable in the reversible inhibition of the binding activity at detergent concentrations above or around the critical micellar concentration, as those needed for the solubilization of membrane pellets. Only upon dilution of the detergent was the binding activity recovered in the soluble extract. With detergent concentrations above the optimum found for solubilization of binding activity (0.75% w/v), there was inactivation of an increasingly larger fraction of the solubilized binding activity. This second observation may point to the existence of a multimeric receptor complex which is progressively dissociated into inactive subunits by high detergent concentrations or may underscore a more complex denaturation kinetics of the binding moiety by the detergent. A similar solubilization profile by CHAPS was also reported for the neurotensin receptor (Mazella et al., 1988) and the interleukin 1 receptor (Paganelli et al., 1987).

Proof of solubilization of CHAPS-extracted bombesin/GRP receptor was obtained by both floating in high centrifugal force field and partitioning between void and included volume in molecular sieving chromatography. The apparent molecular size calculated for the solubilized bombesin/GRP receptor by the gel filtration experiments was 200000 daltons, significantly larger than the 75 kDa displayed in SDS–polyacrylamide gel electrophoresis by the protein labeled with cross-linking experiments employing radiolabeled GRP. This discrepancy may be explained by differential interaction of the same protein with nondenaturing and denaturing detergents, or alternatively by the existence of a homo- or heterooligomeric receptor whose components are held together by noncovalent interactions. These components can be a G-protein complex (Letterio et al., 1986; Fischer & Schonbrunn, 1988), a protein-tyrosine kinase (Cirillo et al., 1986; Gaudino et al., 1988), or other unidentified molecules.

The identity of the solubilized binding activity with the bombesin/GRP receptor analyzed in membrane pellets was proven by (1) specific solubilization of the activity only from cells or tissues known to express the bombesin/GRP receptor, (2) similarity of the dissociation constants for ligand binding of the solubilized activity and the membrane-bound receptor, (3) similarity of ionic effects on the binding of bombesin to both membrane-bound and solubilized receptor, and (4) concordance in the relative binding affinities of a spectrum of agonists and one antagonist.

Lipid requirement of the binding may also reflect the described membrane interaction of some of the peptides of the bombesin family. These and other neuropeptides, like opioid peptides and neurokinins, insert their segment responsible for receptor binding (the C-terminal in the case of BN) into the hydrophobic compartments of lipid membranes as perpendicularly oriented helical domains (Cavatorta et al., 1986; Erne

& Schwyzer, 1987). This ligand–lipid bilayer interaction may effect the proper orientation of the ligand and its recognition of the receptor binding site.

A second observation concerning the interaction of bombesin peptides with their receptor was the concentration-dependent inhibition of binding by monovalent cations. Previously it was demonstrated that cations inhibit binding to other neuropeptide receptors, including the neurotensin receptor (Mazella et al., 1988) and the gonadotropin-releasing hormone receptor (Hazum, 1987). This result may be due to impairment of ionic interactions between a negatively charged residue in the receptor and the positively charged arginine residue in the ligand. Also, salts may induce destabilization of the receptor–lipid complex.

In conclusion, we have characterized the binding of peptides of the bombesin/GRP family to their receptor in terms of physicochemical, kinetics, and equilibrium-binding parameters. A protocol was then developed to solubilize the receptor in such a way to preserve its binding characteristics. This solubilization protocol, producing a stable soluble bombesin/GRP receptor, will be a valuable tool for the purification and detailed molecular characterization of this molecule.

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REFERENCES

Anastasi, A., Erspamer, V., & Bucci, M. (1971) Isolation and structure of bombesin and alytesin, two analogous active peptides from the skin of the European amphibians Bombina and Alytes. *Experientia 27*, 166–7.

Bristow, D. R., & Martin, I. L. (1987) Solubilization of the γ-aminobutyric acid/benzodiazepine receptor from rat cerebellum: optimal preservation of the modulatory responses by natural brain lipids. *J. Neurochem. 49*, 1386–93.

Bruns, R. F., Lawson-Wendling, K., & Pugsley, T. A. (1983) A rapid filtration assay for soluble receptors using polyethylenimine-treated filters. *Anal. Biochem. 132*, 74–81.

Carney, D. N., Cuttitta, F., Moody, T. W., & Minna, J. D. (1987) Selective stimulation of small cell lung cancer clonal growth by bombesin and gastrin-releasing peptide. *Cancer Res. 47*, 821–5.

Cavatorta, P., Faruggia, G., Masotti, L., Sartor, G., & Szabo, A. G. (1986) Conformational flexsibility of the hormonal peptide bombesin and its interaction with lipids. *Biochem. Biophys. Res. Commun. 141*, 99–105.

Cirillo, D. M., Gaudino, G., Naldini, L., & Comoglio, P. (1986) Receptor for bombesin with associated tyrosine kinase activity. *Mol. Cell. Biol. 6*, 4641–9.

Cuttitta, F., Carney, D. N., Mulshine, J., Moody, T. W., Fedorko, J., Fischler, A., & Minna, J. D. (1985) Bombesin-like peptides can function as autocrine growth factors in human small-cell lung cancer. *Nature 316*, 823–6.

Erne, D., & Schwyzer, R. (1987) Membrane structure of bombesin studied by infrared spectroscopy. Prediction of membrane interactions of gastrin-releasing peptide. *Biochemistry 26*, 6316–9.

Fischer, J. B., & Schonbrunn, A. (1988) The bombesin receptor is coupled to a guanine nucleotide-binding protein which is insensitive to pertussis and cholera toxins. *J. Biol. Chem. 263*, 2808–16.

Guadino, G., Cirillo, D., Naldini, L., Rossino, P., & Comoglio, P. (1988) Activation of the protein-tyrosine kinase associated with the bombesin receptor complex in small cell lung carcinomas. *Proc. Natl. Acad. Sci. U.S.A. 85*, 2166–70.

Gmerek, D. E., & Cowan, A. (1982) Classification of opiods on the basis of their ability to antagonize bombesin-induced grooming in rats. *Life Sci. 31*, 2229–32.

Hazum, E. (1987) Binding properties of solubilized gonadotropin-releasing hormone receptor: role of carboxylic groups. *Biochemistry 26*, 7011–4.

Hjelmeland, L. M., & Chrambach, A. (1984) Solubilization of functional membrane proteins. Methods *Enzymol. 104*, 305–18.

Jensen, R. T., Moody, T. W., Pert, C., Rivier, J. E., & Gardner, J. D. (1978) Interaction of bombesin and litorin with specific membrane receptors on pancreatic acinar cells. *Proc. Natl. Acad. Sci. U.S.A. 75*, 6139–43.

Jensen, R. T., Jones, S. W., Folkers, K., & Gardner, J. D. (1984) A synthetic peptide that is a bombesin receptor antagonist. *Nature 309*, 61–3.

Kris, R. M., Hazan, R., Villines, J., Moody, T. W., and Schlessinger, J. (1987) Identification of the bombesin receptor on murine and human cells by cross-linking experiments. *J. Biol. Chem. 262*, 11215–20.

Letterio, J. J., Coughlin, S. R., & Williams, L. T. (1986) Pertussis toxin-sensitive pathway in the stimulation of c-myc expression and DNA synthesis by bombesin. *Science 234*, 1117–9.

Levitzki, A. (1985) Reconstitution of membrane receptor systems. *Biochim. Biophys. Acta 822*, 127–53.

Mazella, J., Chabry, J., Kirabgi, P., & Vincent, J. P. (1988) Solubilization and characterization of active neurotensin receptors from mouse brain. *J. Biol. Chem. 263*, 144–9.

McDonald, T. J., Jornvall, H., Nilsson, G., Vagne, M., Ghatei, M., Bloom, S. R., & Mutt, V. (1979) Characterization of a gastrin releasing peptide from porcine non-antral gastric tissue. *Biochem. Biophys. Res. Commun. 90*, 227–33.

Paganelli, K. A., Stern, A. S., & Kilian, P. L. (1987) Detergent solubilization of the interleukin 1 receptor. *J. Immunol. 138*, 2249–53.

Rozengurt, E., & Sinnett-Smith, J. (1983) Bombesin stimulation of DNA synthesis and cell division in cultures of Swiss 3T3 cells. *Proc. Natl. Acad. Sci. U.S.A. 80*, 2936–40.

Stuckey, J. A., Gibbs, J., & Smith, G. P. (1982) Lateral hypothalmic injection of bombesin decreases food intake in rats. *Brain Res. Bull. 8*, 617–21.

Sunday, M. E., Kaplan, L. M., Moroyama, E., Chin, W. W., & Spindel, E. R. (1988) Gastrin-releasing peptide (mammalian bombesin) gene expression in health and disease. *Lab. Invest. 59*, 5–24.

Swope, S. L., & Schonbrunn, A. (1987) Characterization of ligand binding and processing by bombesin receptors in an insulin-secreting cell line. *Biochem. J. 247*, 731–8.

Tache, Y., & Gunion, M. (1985) Central nervous system action of bombesin to inhibit gastric acid secretion. *Life Sci. 37*, 115–23.

Willey, J. C., Lechner, J. F., & Harris, C. C. (1984) Bombesin and the C-trerminal tetradecapeptide of gastrin-releasing peptide are growth factors for normal human bronchial epithelial cells. *Exp. Cell. Res. 153*, 245–8.

Zachary, I., & Rozengurt, E. (1985) High-affinity receptors for peptides of the bombesin family in Swiss 3T3 cells. *Proc. Natl. Acad. Sci. U.S.A. 82*, 7616–20.

Zachary, I., & Rozengurt, E. (1987) Identification of a receptor for peptides of the bombesin family in Swiss 3T3 cells

by affinity cross-linking. *J. Biol. Chem. 262*, 3947-50.
Zachary, I., Woll, P. J., & Rozengourt, E. (1987) A role for neuropeptides in the control of cell proliferation. *Dev. Biol. 124*, 295-308.
Zarbin, M. A., Kuhar, M. J., O'Donohue, T. L., Wolf, S. S., & Moody, T. W. (1985) Autoradiographic localization of (^{125}I-Tyr^4)bombesin-binding sites in rat brain. *J. Neurosci. 5*, 429-37.

Effect of Guanine Nucleotides on the Hydrophobic Interaction of Tubulin†

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ABSTRACT: The influence of guanine nucleotides on the binding of tubulin to hydrophobic components is investigated. Tubulin binds to a hydrophobic phenyl-Sepharose gel in a reversible, nucleotide-dependent way. Assembly-competent tubulin is released with ion-free water as eluent. It contains one guanosine triphosphate per dimer. More denatured tubulin needs a mixture of ethanol–water to elute. Consequently, hydrophobic interaction chromatography over phenyl-Sepharose represents an easy method for preparing polymerizable tubulin free of nucleotides at the exchangeable sites. While, in the absence of guanine nucleotide, the binding of tubulin to phenyl-Sepharose is rapid and immediately reversible on nucleotide addition, the binding of the nucleotide-dependent hydrophobic sites of tubulin to 1,8-ANS is slow, and its dissociation on nucleotide addition is poor. No differences are observed between the shielding of hydrophobic sites in the presence of GTP or GDP. Neither inorganic phosphate nor AlF_4^- is found to directly influence guanine nucleotides in their ability to shield hydrophobic sites.

Both α- and β-tubulin subunits are anionic polypeptides whose carboxyl-terminal sequences are particularly rich in acidic amino acids. These regions play an important regulatory role in the assembly of microtubules through the binding of microtubule-associated proteins (MAPs)[1] (Serrano et al., 1984; Littauer et al., 1986).

Less is known about the nature of the tubulin–tubulin interactions that lead to the formation of microtubules. An involvement of hydrophobic interactions can be argued by the fact that dimeric tubulin contains extensive and easily accessible regions capable of hydrophobic interaction (Andreu, 1982; Horowitz et al., 1984; Kocha et al., 1986; Prasad et al., 1987; Stephens, 1988). Association of strongly binding hydrophobic reagents inhibits self-assembly (Horowitz et al., 1984; Andreu & Timasheff, 1982). Also the increase of the enthalpy and entropy, and the negative heat capacity change accompanying the formation of microtubules (Lee & Timasheff, 1977; Hinz & Timasheff, 1986), indicates that the largest contribution of these thermodynamic properties is given by the removal of nonpolar protein surfaces from water.

Although the hydrophobic nature of proteins can be importantly influenced by their natural ligands (Desmet et al., 1987), up to now, no research on the effect of natural tubulin ligands, such as GTP and GDP, has been done. In the present study we clearly demonstrate that the removal of exchangeable guanine nucleotides strongly influences the hydrophobic behavior of tubulin and that assembly-competent tubulin, containing one guanosine triphosphate per dimer, can be obtained by hydrophobic interaction chromatography on phenyl-Sepharose. As microtubules are stabilized by inorganic phosphate and by its structural analogue AlF_4^- (Carlier et al., 1988), we also investigated whether these anions, together with guanine nucleotides, can modulate the hydrophobic behavior of tubulin.

Materials and Methods

Pig brains are obtained from freshly slaughtered animals and kept in ice-cold buffer containing 100 mM MES–KOH, 1 mM $MgSO_4$, and 1 mM EGTA, pH 6.5. The brains are used within 1 h after slaughter. The nucleotides GTP and GDP are purchased from Boehringer. 1,8-ANS is obtained from Molecular Probes and phenyl-Sepharose CL-4B from Pharmacia.

Preparation of Tubulin and Microtuble-Associated Proteins. Pig brain tubulin is prepared by two polymerization cycles, as described by Shelanski et al. (1973). The pellet obtained after the second polymerization is resuspended in a buffer containing 100 mM MES–KOH, 0.1 mM GTP, 1 mM $MgSO_4$, and 1 mM EGTA, pH 6.5, and stored in liquid nitrogen. Just before use, tubulin is purified from microtubule-associated proteins by chromatography on phosphocellulose. The elution buffer consists of MM buffer, containing in addition 40 mM KCl (or 15 mM K_2SO_4) and 0.05 mM GTP. The fraction of microtubule-associated proteins is eluted with 0.8 M NaCl and chromatographed over Sephadex G-25 with MM buffer containing 15 mM K_2SO_4. Protein concentrations are determined by the method of Folin–Ciocalteu, using bovine serum albumin as a standard. In order to calculate molar concentrations, the molecular weight of a tubulin dimer is taken to be 100 000.

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[1] Abbreviations: 1,8-ANS, 8-anilinonaphthalene-1-sulfonate; GTP, guanosine 5′-triphosphate; GDP, guanosine 5′-diphosphate; MES, 4-morpholineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; MAPs, microtubule-associated proteins; MM buffer, 10 mM MES–KOH (pH 6.5) and 1 mM $MgSO_4$; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; PC-tubulin, phosphocellulose-purified tubulin.

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