Obara, M., Kang, S. M., & Yamada, K. M. (1988) *Cell 53*, 649–657.
Phillips, D. R., & Agin, P. P. (1977) *J. Biol. Chem. 252*, 2121–2126.
Pierschbacher, M. D., & Ruoslahti, E. (1984) *Nature 309*, 30–33.
Plow, E. F., & Ginsberg, M. H. (1981) *J. Biol. Chem. 256*, 9477–9482.
Pytela, R., Pierschbacher, M. D., Ginsberg, M. A., Plow, E. F., & Ruoslahti, E. (1986) *Science 231*, 1559–1562.
Ruggeri, Z. M., Bader, R., & DeMarco, L. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 6038–6041.
Sambrook, J., Fritsch, E. F., & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Solowska, J., Guan, J. L., Marcantonio, E. E., Trevithick, J. L., Buck, C. A., & Hynes, R. O. (1989) *J. Cell Biol. 109*, 853–861.
Tarentino, A. L., & Maley, T. (1974) *J. Biol. Chem. 249*, 811–817.
Thiagarajan, P., & Kelly, K. L. (1988) *J. Biol. Chem. 263*, 3035–3038.
Troesch, A., Duperray, A., Polack, B., & Marguerie, G. (1990) *Biochem. J. 268*, 129–133.

Solubilization and Molecular Characterization of Active Galanin Receptors from Rat Brain†

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Received September 13, 1991; Revised Manuscript Received December 10, 1991

ABSTRACT: Galanin receptors were solubilized from rat brain using the zwitterionic detergent 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid (CHAPS). Binding of ^{125}I-galanin to the soluble fraction was time- and temperature-dependent, saturable, and reversible. Scatchard analysis of binding data indicated that the soluble extract contained a single class of galanin binding sites with a K_d of 0.8 nM and a B_{max} of 26 fmol/mg of protein. Unlabeled galanin and its fragments galanin(2–29) and galanin(1–15) antagonized the binding of ^{125}I-galanin to CHAPS-solubilized extracts with relative potencies similar to those observed with membrane receptors. Galanin(3–29) was found inactive. Binding of ^{125}I-galanin to CHAPS extracts was inhibited by guanine nucleotides with the following rank order of potency: GMP-P(NH)P > GTP > GDP. Molecular analysis of the soluble galanin receptor by covalent cross-linking of ^{125}I-galanin to CHAPS extracts using disuccinimidyl tartarate and further identification on SDS–PAGE indicated that the soluble galanin binding site behaves as a protein of M_r 54 000. After incubation of CHAPS extracts with ^{125}I-galanin, gel filtration on Sephacryl S-300 followed by ultracentrifugation on sucrose density gradient revealed a binding component with the following hydrodynamic parameters: Stokes radius, 5 nm; $s_{20,w}$, 4.5 S; M_r, 98 000; frictional ratio, 1.6. GMP-P(NH)P treatment of CHAPS extracts gave rise to a molecular form with the following characteristics: Stokes radius, 4 nm; $s_{20,w}$, 3.3 S; M_r, 57 000; frictional ratio, 1.4. Assuming one molecule of ^{125}I-galanin (M_r 3000) is bound per molecule of receptor, these data suggest that brain galanin receptor consists of a M_r 54 000 protein associated with the α subunit of a G protein. The availability of this CHAPS-soluble receptor from rat brain represents a major step toward the purification of this newly characterized receptor.

Galanin, a 29 amino acid peptide isolated from the porcine intestine (Tatemoto et al., 1983), is widely distributed in the central and peripheral nervous system of numerous species (Ch'ng et al., 1985). It exerts various biological effects, including inhibition of transmitter release from neurons (Rökaeus, 1987; Fisone et al., 1987), contraction of smooth muscle (Rökaeus, 1987; Ekblad et al., 1985), control of hypothalamic–anterior pituitary functions (Ottlecz et al., 1988), and regulation of gastric and pancreatic endocrine secretions (Kwok et al., 1988; McDonald et al., 1985; Silvestre et al., 1987). According to the potent inhibitory effect of galanin on insulin secretion (McDonald et al., 1985), the first galanin receptors to be discovered originated from a hamster-transplantable pancreatic β-cell tumor (Amiranoff et al., 1987). Using a rat insulin-secreting pancreatic β-cell line in culture, Rin m5F, we demonstrated thereafter that the galanin receptor behaves as a glycoprotein coupled to a pertussis toxin sensitive G_i[1] protein mediating inhibition of adenylyl cyclase in the plasma membrane (Amiranoff et al., 1988, 1989a; Lagny-Pourmir et al., 1989a). Meanwhile, galanin receptors were discovered in rat brain membranes by our group (Servin et

† This work was supported in part by the Fondation pour la Recherche Médicale (postdoctoral fellowship to Y.C.).
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[1] Abbreviations: CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; DST, disuccinimidyl tartarate; CHS, cholesteryl hemisuccinate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PMSF, phenylmethanesulfonyl fluoride; TLCK, N^{α}-*p*-tosyl-L-lysine chloromethyl ketone; GMP-P(NH)P, guanyl-5′-yl imidodiphosphate; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; G_i, inhibitory regulatory GTP binding protein of adenylyl cyclase.

0006-2960/92/0431-2415$03.00/0

al., 1987) and further characterized in hippocampus by others (Fisone et al., 1989a). Interestingly, remarkable similarities between pancreatic and brain galanin receptors could be noticed, e.g., the high affinity of both receptors for galanin (Amiranoff et al., 1987; Lagny-Pourmir et al., 1989a; Servin et al., 1987; Fisone et al., 1989a), their coupling with a pertussis toxin sensitive G_i protein (Amiranoff et al., 1988; Fisone et al., 1989a), and the structural requirement of their interaction with galanin and galanin fragments (Amiranoff et al., 1989b; Lagny-Pourmir et al., 1989b; Fisone et al., 1989b). Affinity labeling experiments also gave evidence that these galanin receptor binding sites identified in brain (Servin et al., 1987) and in pancreatic β cells (Amiranoff et al., 1987, 1989a; Lagny-Pourmir et al., 1989a) as well as those recently characterized in a pancreatic δ-cell line (Amiranoff et al., 1991) have an identical structure.

These preliminary insights on galanin receptors were gained by cross-linking experiments, i.e., in denaturing detergent conditions by SDS–PAGE analysis of membrane components covalently labeled with ^{125}I-galanin. Therefore, they do not document the nature and molecular form(s) of receptors in a functional state. Using rat brain as a receptor source, we now report the successful solubilization of active galanin receptors by the nondenaturing detergent CHAPS. Thereby, we have been able to analyze their hydrodynamic properties by molecular sieving and sucrose density gradient ultracentrifugation. We provide evidence for the solubilization of a M_r 98 000 complex from which the M_r 54 000 galanin receptor binding site can be released when guanine nucleotides are present.

Materials and Methods

Materials. Synthetic galanin, bacitracin, leupeptin, GTP, GDP, GMP-P(NH)P, CHAPS, TLCK, and PMSF were obtained from Sigma; SDS–PAGE chemicals and marker proteins were from Bio-Rad; DST was from Pierce; Sephadex G-25 and Sephacryl S-300 were from Pharmacia. Synthetic galanin was radioiodinated with ^{125}I (Amersham, England) using the chloramine T method (Hunter & Greenwood, 1962), at a specific activity of 700 Ci/mmol. As verified earlier (Lagny-Pourmir et al., 1989a), iodination of galanin by this method does not alter the biological activity of the peptide. The galanin fragments galanin(2–29), galanin(3–29), and galanin(1–15) were kindly provided by Professor N. Yanaihara (Shizuoka, Japan).

Preparation of Rat Brain Membranes. Rat brain membranes were prepared as previously reported (Servin et al., 1987). Briefly, male Wistar rats (180–200 g) were decapitated, and brain was quickly removed and homogenized using a Waring Blendor for 2 min in an ice-cold Tris-HCl buffer (50 mM, pH 7.5) containing NaCl (100 mM), $MgCl_2$ (5 mM), EDTA (1 mM), bacitracin (100 μg/mL), TLCK (10 μg/mL), PMSF (100 μM), and leupeptin (10 μg/mL). After centrifugation for 10 min at 20000*g*, the pellet was washed twice in Hepes buffer (20 mM, pH 7.5), and the final material containing the crude membranes was stored at −80 °C until use.

Membrane Solubilization. In optimal conditions, solubilization was carried out by incubating membranes (7.5 mg/mL) in Hepes buffer (20 mM, pH 7.5) containing CHAPS (30 mM), CHS (1.8 mM), PMSF (0.1 mM), 0.01% (w/v) sodium azide, 30% (v/v) glycerol, and KCl (25 mM) for 30 min at 0 °C. After a 2-fold dilution with the same CHAPS-free buffer, the suspension was centrifuged for 60 min at 100000*g* (4 °C). The supernatant was then removed and stored at −80 °C. In those conditions, the yield of membrane protein solubilization was estimated at 40%. The ability of the solubilized brain material to bind ^{125}I-galanin was found to be stable after storing for 3 days at 4 °C and even for 1 month at −80 °C. Protein concentration was determined by the Bio-Rad procedure (Bradford, 1976) with bovine serum albumin as a standard.

Binding and Cross-Linking of ^{125}I-Galanin to Membranes. Binding of ^{125}I-galanin to rat brain membranes was performed as described in detail elsewhere (Servin et al., 1987). For affinity cross-linking, the labeled membranes were resuspended in 1 mL of 20 mM Hepes (pH 7.5) containing DST at the final concentration of 1 mM. The cross-linking reaction was carried out for 20 min at 4 °C and stopped by centrifugation at 20000*g* for 10 min. Membranes were then solubilized in a SDS-containing medium before SDS–PAGE (see below).

Binding and Cross-Linking of ^{125}I-Galanin to CHAPS Extracts. ^{125}I-galanin binding to solubilized material was studied essentially as described for membranes. In standard conditions, solubilized material was incubated for 4 h at 15 °C in 20 mM Hepes buffer (pH 7.5) containing 2% (w/v) bovine serum albumin and 1 mg/mL bacitracin. At the end of incubation, separation of ^{125}I-galanin bound to soluble receptors from the free ligand was achieved by glass fiber filtration using Whatman GF/C filters pretreated with 0.5% (v/v) poly(ethylenimine) in water (Bruns et al., 1983). The filters were then washed twice with 5 mL of ice-cold 10 mM Hepes buffer (pH 7.5), and the radioactivity retained on filters was counted. Specific binding was calculated as the difference between the amount of ^{125}I-galanin bound in the absence (total binding) or presence (nonspecific binding) of an excess (10^{-7} M) of native galanin. Nonspecific binding represented 30% of total binding and specific binding about 3% of total radioactivity. For cross-linking experiments, free ^{125}I-galanin and ^{125}I-galanin–receptor complexes were first separated by gel filtration on a Sephadex G-25 (superfine) column (15 × 0.9 cm). The column run at 1 mL/min was equilibrated and eluted at 4 °C with Hepes buffer (20 mM, pH 7.5) containing CHAPS (1 mM), KCl (12.5 mM), $MgCl_2$ (5 mM), and PMSF (0.1 mM). One milliliter of the void volume of the G-25 column that contained ^{125}I-galanin–receptor complexes was then incubated with 1 mM DST. After 20 min at 4 °C, the cross-linking reaction was stopped by adding 20 μL of ice-cold 1 M Tris (pH 6.8). The samples were then prepared for SDS–PAGE as described (Amiranoff et al., 1989a).

SDS–Polyacrylamide Gel Electrophoresis. Cross-linked materials were analyzed on SDS–PAGE according to the Laemmli method (Laemmli et al., 1970), using a 5% acrylamide stacking gel and a 12% polyacrylamide slab gel as described (Amiranoff et al., 1989a). The dried gels were exposed for 1 week at −80 °C to a Trimax Type XM film (3M) with a 3M Trimax intensifying screen. Gels were calibrated with the following proteins of known molecular weight: myosin (200 000), phosphorylase *b* (92 500), bovine serum albumin (68 000), ovalbumin (43 000), carbonic anhydrase (29 000), lactoglobulin (18 400), and lysozyme (14 300).

Gel Filtration of Solubilized Materials. CHAPS extracts (1.5–2 mg/mL) were chromatographed on a Sephacryl S-300 column (0.9 × 15 cm) equilibrated and eluted with Hepes buffer (20 mM, pH 7.5) as described above. Fractions of 0.5 mL were collected at a flow rate of 0.5 mL/min. Specific ^{125}I-galanin binding was measured in each fraction as described above. Two sets of separate experiments were also conducted: (1) CHAPS extracts (1.5–2 mg of protein/mL) were first incubated with ^{125}I-galanin (0.5 nM) in the absence or presence

of 10^{-7} M native galanin for 4 h at 15 °C as described above. Free and receptor-bound ^{125}I-galanins were separated by gel filtration on a Sephadex G-25 column, and the void volume of this column was submitted to gel filtration on the Sephacryl S-300 in the above-described conditions. (2) Membranes were incubated with ^{125}I-galanin (0.5 nM) in the absence or presence of 10^{-7} M native galanin for 90 min at 15 °C. After centrifugation (20000*g*, 10 min), membrane proteins were extracted by incubation with 30 mM CHAPS for 30 min at 0 °C. After gel filtration of CHAPS extracts on Sephadex G-25, the void volume was then submitted to gel filtration on the Sephacryl S-300 column. The S-300 column was calibrated under conditions used for analyzing solubilized receptors, with the following marker proteins of known Stokes radius: apoferritin (6.1 nm), catalase (5.2 nm), γ-globulin (5.1 nm), bovine serum albumin (3.55 nm), hemoglobin (3.2 nm), and myoglobin (1.9 nm). Blue dextran 2000 and $K_3Fe(SCN)_6$ were used to determine the void volume (V_0) and the total liquid volume (V_t) of the column, respectively. The elution volumes of receptor and marker proteins were expressed in term of $K_{av} = (V_e - V_0)/(V_t - V_0)$ where V_e is the elution volume of the protein considered. In order to estimate the Stokes radius of galanin receptors, K_{av} was plotted against the known Stokes radius of the above-mentioned markers.

Sucrose Density Gradient Ultracentrifugation. CHAPS extracts (2 mg/mL) were first incubated with ^{125}I-galanin for 4 h at 15 °C. The apparent sedimentation coefficient of ^{125}I-galanin–receptor complexes was further determined by ultracentrifugation on a sucrose density gradient of the material eluted in the peaks obtained by gel filtration on Sephacryl S-300 as described above. Linear 5–20% sucrose density gradients (4 mL) were prepared from 5 and 20% (w/v) sucrose solution in H_2O or D_2O (98% D_2O) containing 20 mM Hepes (pH 7.5), 12.5 mM KCl, 5 mM $MgCl_2$, and 1 mM CHAPS. Samples (0.2 mL) were layered onto the top of the gradient, and centrifugation was performed at 4 °C for 16 h at 43 000 or 55 000 rpm for H_2O or D_2O sucrose gradients, respectively. Calibration curves for the determination of apparent sedimentation coefficients were constructed according to the method of Martin and Ames (1961) using the following protein markers: cytochrome *c* ($s_{20,w}$ = 1.17 S); bovine serum albumin ($s_{20,w}$ = 4.6 S); bovine γ-globulin ($s_{20,w}$ = 7.2 S); catalase from bovine liver ($s_{20,w}$ = 11.4 S). After centrifugation, fractions of 120 μL were collected from the top of each gradient.

Data Analysis. Analysis of saturation and competition experiments was performed by the EBDA/LIGAND program.

Results

Solubilization of Rat Brain Galanin Receptor. To determine the opimal conditions for solubilization of active galanin receptors, solubilization of rat brain membranes was carried out using a fixed concentration of membrane protein (8 mg/mL) and increasing concentrations of CHAPS, a detergent commonly used to solubilize peptide receptors in an active state (Liscia et al., 1982; Couvineau et al., 1986; Voisin et al., 1991). As shown in Figure 1 (left), CHAPS solubilized membrane protein in a dose-dependent manner with a maximal efficiency observed with 30 mM detergent. Accordingly, binding of ^{125}I-galanin to the solubilized material increased with the CHAPS concentration. Solubilization of increasing concentrations of membrane proteins was then tested in the presence of 30 mM CHAPS (Figure 1, right). As expected, binding of ^{125}I-galanin to the soluble material increased with membrane protein concentrations. A plateau of binding was observed for a membrane protein concentration close to 8 mg/mL. Using 30 mM CHAPS and 8 mg/mL membrane protein concentration as optimal conditions for solubilization, specific binding of ^{125}I-galanin to CHAPS extracts was found to be proportional to the incubated solubilized protein concentrations ranging from 0.7 to 3.5 mg of protein/mL (not shown).

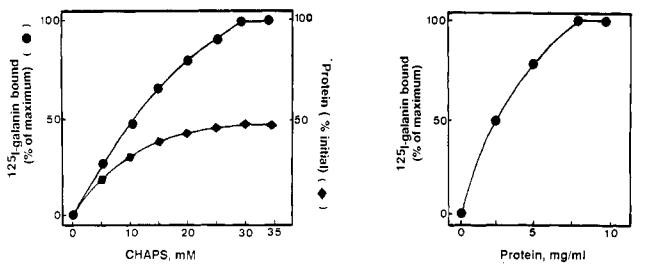

FIGURE 1: Effects of CHAPS and membrane protein concentration on the solubilization of galanin receptors. (Left) Membranes (8 mg of protein/mL) were incubated for 30 min at 0 °C with the indicated concentrations of CHAPS. After centrifugation for 60 min at 100000*g*, the specific binding of ^{125}I-galanin (●) in the soluble material was measured. Proteins in CHAPS extract (♦) after solubilization are expressed as the percent of initial protein content in membranes before solubilization. (Right) Membranes (at the indicated concentrations) were incubated with 30 mM CHAPS. The specific binding of ^{125}I-galanin was then measured in the soluble fraction. Each point is the mean of triplicate determinations of one typical experiment. Another experiment gave similar results.

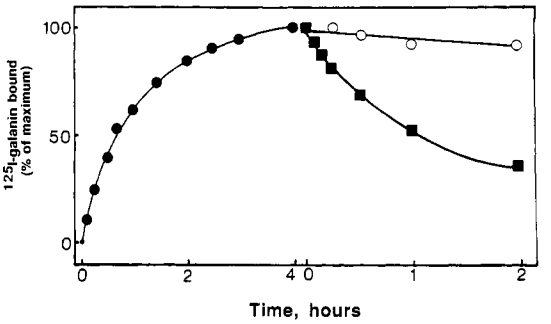

FIGURE 2: Association and dissociation kinetics of specific ^{125}I-galanin binding to CHAPS-solubilized extracts. Association of the tracer to CHAPS extracts was performed at 15 °C during the indicated period. Dissociation of bound ^{125}I-galanin was measured after addition of 50 μL of incubation buffer in the absence (○) or presence (■) of 10^{-5} GMP-P(NH)P. Each point is the mean of triplicate determinations in one typical experiment. Two other experiments gave similar results.

Kinetics, Stoichiometry, and Specificity of the Interaction of Galanin with Solubilized Receptors. Specific binding of ^{125}I-galanin to soluble receptors was time- and temperature-dependent. At 30 °C, it rapidly reached a maximum after 1 h and then decreased progressively during the following 2 h (not shown). At 15 °C, binding of the tracer was slower but reached a plateau after 4 h (Figure 2). Under optimal conditions (4 h at 15 °C), binding of galanin to soluble receptors was saturable (Figure 3). Native galanin concentrations ranging from 10^{-11} to 10^{-8} M competitively inhibited the binding of ^{125}I-galanin to soluble receptors (Figure 3). Scatchard analysis of the data (Figure 3, inset) gave a straight line indicating the existence of a single class of solubilized galanin binding sites. From five experiments, the dissociation constant (K_d) was estimated at 0.80 ± 0.03 nM and the concentration of binding sites at 26 ± 3 fmol/mg of protein.

The integrity of the molecule of galanin is crucial for its binding activity to brain membrane receptor (Lagny-Pourmir et al., 1989b). The specificity of soluble galanin receptors was studied by testing the ability of galanin fragments, e.g., Gal(3–29), Gal(2–29), and Gal(1–15), to inhibit the binding of ^{125}I-galanin to solubilized receptors (Figure 4). While Gal(3–29) did not alter specific ^{125}I-galanin binding to solu-

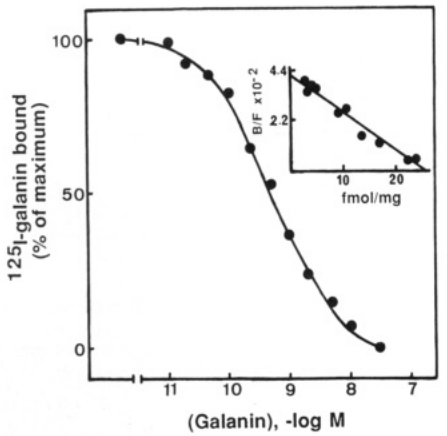

FIGURE 3: Competitive inhibition of ^{125}I-galanin binding to soluble receptors by native galanin. CHAPS extracts (2 mg/mL) were incubated with ^{125}I-galanin and increasing concentrations of native galanin under standard conditions specified under Materials and Methods. Specific binding was expressed as the percentage of maximum binding measured in the presence of tracer alone. Inset: Scatchard analysis of the galanin binding data. Each point is the mean of triplicate measurements within a typical experiment.

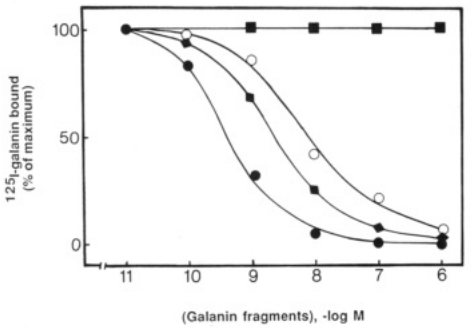

FIGURE 4: Effect of galanin fragments on the binding of ^{125}I-galanin to CHAPS extracts. CHAPS extracts (2 mg of protein/mL) were incubated with ^{125}I-galanin and increasing concentrations of galanin(1–29) (●), galanin(2–29) (♦), galanin(3–29) (■), and galanin-(1–15) (○). Results are expressed as the percentage of maximal specific binding measured in the presence of tracer alone. Each point is the mean of triplicate measurements in one typical experiment. Another experiment gave similar results.

bilized receptors, other fragments competitively inhibited tracer binding with the following order of potency: Gal(1–29) > Gal(2–29) > Gal(1–15) (Figure 4).

Molecular Characterization of the Solubilized Brain Galanin Receptor. Affinity cross-linking of ^{125}I-galanin to CHAPS extracts of rat brain membranes was first performed. After submission of the cross-linked materials to SDS–PAGE (see Materials and Methods), the resulting autoradiography revealed a single labeled band corresponding to the migration of a protein of M_r 57 000 (Figure 5, lane 1). The labeling of this band was totally abolished by incubating the CHAPS extract with an excess of native galanin (Figure 5, lane 2), indicating that the labeled protein does indeed represent the soluble galanin receptor. A similar pattern was obtained when cross-linking of ^{125}I-galanin to rat brain membranes was performed (Figure 5, lanes 3 and 4). Assuming one molecule of ^{125}I-galanin (M_r 3000) is linked per molecule of receptor, the intrinsic molecular weight of the soluble galanin receptor binding site is 54 000.

Further experiments were carried out to determine the hydrodynamic properties of solubilized galanin receptors. CHAPS extracts of brain membranes were chromatographed on a Sephacryl S-300 column, and aliquots of the collected fractions were tested for their ability to interact with ^{125}I-

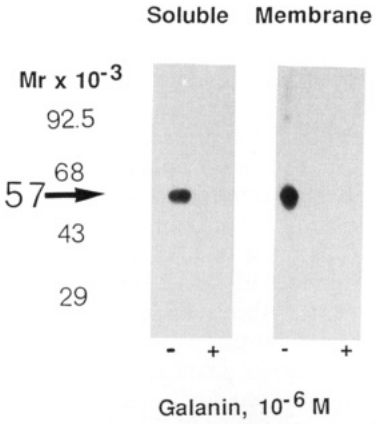

FIGURE 5: Affinity cross-linking of ^{125}I-galanin to CHAPS extracts or membranes. CHAPS extracts (2 mg of protein/mL) or membranes (0.25 mg of protein/mL) were incubated with ^{125}I-galanin without or with 1 μM unlabeled galanin. ^{125}I-Galanin–receptor complexes were separated from free ^{125}I-galanin by gel filtration on a Sephadex G-25 column, then treated with 1 mM DST, and submitted to SDS–PAGE. For detail, see Materials and Methods.

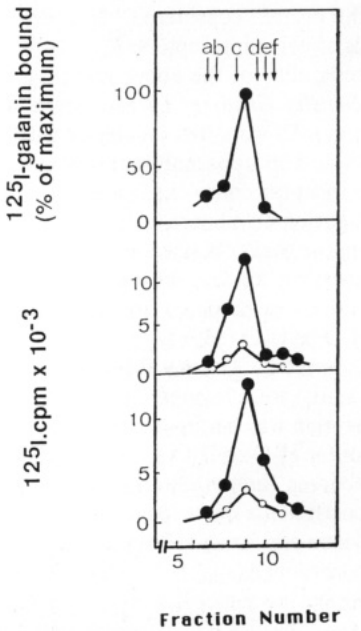

FIGURE 6: Gel filtration profiles of solubilized galanin receptors. (Top) CHAPS extracts (2 mg of protein/mL) were loaded on a Sephacryl S-300 column (0.9 × 15 cm). Specific binding of ^{125}I-galanin was measured in each fraction (●) as described under Materials and Methods. Results are expressed as the percent of maximal binding measured in fraction 9. (Middle) CHAPS extracts (2 mg of protein/mL) were first incubated with ^{125}I-galanin (10 nM) in the absence (●) or in the presence (○) of 1 μM native galanin as described under Materials and Methods. After 4 h at 15 °C, the incubation medium was loaded onto a Sephadex G-25 column. Aliquots of the void volume were then loaded on the Sephacryl S-300 column. Radioactivity was then measured in each fraction. (Bottom) Brain membranes (2 mg of protein/mL) were first incubated with ^{125}I-galanin (10 nM) in the absence (●) or in the presence (○) of 1 μM native galanin for 90 min at 15 °C. The labeled material was then solubilized in the 30 mM CHAPS buffer and thereafter applied to a G-25 column. Aliquots of the void volume of this column were then loaded onto the S-300 column, and the radioactivity of each fraction was measured. The column was calibrated with proteins of known Stokes radius, e.g., apoferritin (a), catalase (b), γ-globulin (c), bovine serum albumin (d), hemoglobin (e), and myoglobin (f).

galanin in the absence or presence of 1 μM native galanin. The soluble receptor was eluted as a single symmetrical peak around fraction 9 (Figure 6, top), thus corresponding to a protein with a Stokes radius of 5.03 nm. In a separate experiment, the CHAPS extract was preincubated with ^{125}I-

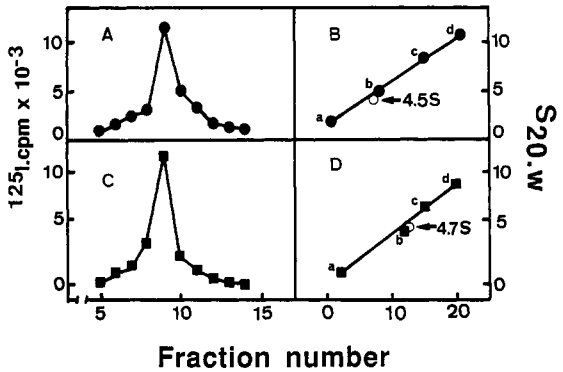

FIGURE 7: Sucrose density gradient ultracentrifugation of ^{125}I-galanin–receptor complexes. The peak obtained after gel filtration on the Sephacryl S-300 column (see Figure 6, middle) was centrifuged through a 5–20% sucrose density gradient in H_2O for 16 h at 43 000 rpm (A) or a 5–20% sucrose gradient in D_2O for 16 h at 55 000 rpm (C). $s_{20,w}$ values of known protein markers centrifuged in H_2O (B) or D_2O (D) were plotted as a function of travel (fraction number). The $s_{20,w}$ value of ^{125}I-galanin–receptor complexes was estimated from this standard curve. The following protein markers were used: a, cytochrome *c*; b, bovine serum albumin; c, bovine γ-globulin; d, catalase. Results represent a typical experiment. Another experiment gave similar results.

galanin for 4 h at 15 °C and then layered onto a Sephadex G-25 column, and ^{125}I-galanin–receptor complexes eluted in the void volume were applied onto the Sephacryl S-300 column. A single major peak of radioactivity was eluted in fraction 9 (Figure 6, middle). When 1 μM galanin was added during preincubation of CHAPS extracts with the tracer, this labeled peak was dramatically decreased (Figure 6, middle). In a third type of experiment, rat brain membranes were preincubated with ^{125}I-galanin before solubilization with CHAPS. As described in the former experiments, the CHAPS extract applied sequentially to a G-25 column and to the S-300 column was eluted as a final radioactive peak in fraction 9 of the latter column (Figure 6, bottom). Again, addition of 1 μM native galanin with tracer during incubation of membranes dramatically decreased the radioactivity in the peak. This series of data strongly supports that prelabeling of soluble or membrane galanin receptors by ^{125}I-galanin does not modify the Stokes radius and consequently the apparent size of the soluble galanin receptor.

The hydrodynamic properties of the soluble galanin receptor were further analyzed by sucrose density gradient ultracentrifugation to provide an estimate of its sedimentation coefficient and molecular weight. For that purpose, the radioactive peak eluted from the Sephacryl S-300 column (see Figure 6, middle) was subjected to ultracentrifugation on a linear 5–20% sucrose gradient made up in H_2O and calibrated with marker proteins of known sedimentation coefficients (Figure 7, top). The radioactive component of Stokes radius of 5.03 nm migrated with an apparent sedimentation coefficient ($s_{20,w}$) of 4.5 S. When ultracentrifugation was performed in D_2O, the apparent sedimentation coefficient was estimated at 4.7 S (Figure 7, bottom). The close similarity between the two apparent sedimentation coefficients in H_2O and D_2O supports the concept that the soluble galanin receptor and the calibrating proteins have a very similar partial specific volume (Clarke, 1975). The frictional ratio and molecular weight of the soluble galanin receptor calculated from gel filtration (Figure 6) and ultracentrifugation (Figure 7) experiments were 1.6 and 98 000, respectively (Table I). The apparent mass of the receptor is thus substantially greater than the 57 000 Da determined in cross-linking studies, suggesting that soluble galanin receptors exist as a multisubunit complex.

Table I: Hydrodynamic Parameters of Galanin–Receptor Complexes Solubilized from Rat Brain

parameters	galanin–receptor complexes: control	GMP-P(NH)P
Stokes radius, α (nm)	5.03	4.00
sedimentation coefficient, $s_{20,w}$ (S)		
in H_2O	4.50	3.30
in D_2O	4.70	ND[c]
$s_{20,w}$ (S)[a]	4.60	
partial specific volume, $\bar{\nu}$ (mL/g)[b]	0.74	0.74
molecular weight, M_r[d]	98000	57000
frictional ratio, f/f_0[e]	1.6	1.4

[a] Values were calculated from all possible paired combinations of data from H_2O and D_2O gradients. [b] The $\bar{\nu}$ of galanin–receptor complexes is assumed to be identical to that of the standard proteins. [c] ND, not determined. [d] Calculated from $M_r = [6\pi N\eta_{20,w}/(1 - \bar{\nu}\rho_{20,w})]s_{20,2}\alpha$ where N is Avogadro's number, $\eta_{20,w}$ is the viscosity of water at 20 °C [0.01002 g/(cm·s)], and $\rho_{20,w}$ is the density of water at 20 °C (0.99823 g/mL). [e] The frictional ratio was calculated assuming a hydration of 0.2 g/g of protein as follows: $f/f_0 = \alpha[4\pi N/3M_r(\bar{\nu} + 0.2)]^{1/3}$.

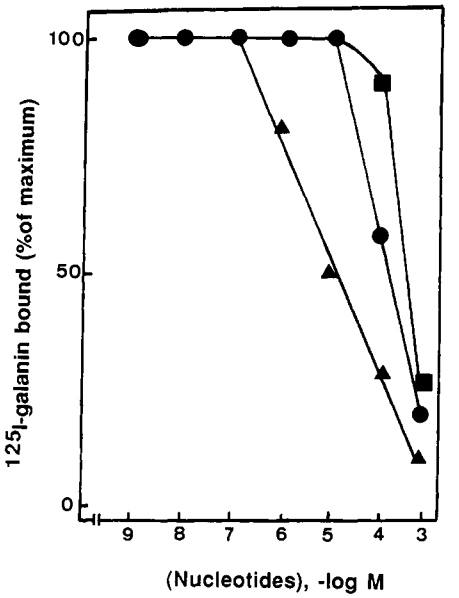

FIGURE 8: Inhibitory effects of guanine nucleotides on the binding of ^{125}I-galanin to CHAPS extracts. CHAPS extracts (2 mg/mL) were incubated with 0.6 nM ^{125}I-galanin and various concentrations of GMP-P(NH)P (▲), GTP (●), or GDP (■). Specific binding was expressed as the percentage of maximal binding measured in the absence of nucleotides. Each point is the mean of triplicate determinations of one typical experiment. Two other experiments gave similar results.

Coupling of Soluble Galanin Receptor with a GTP-Regulatory Protein. As previously demonstrated for other peptide receptors (Couvineau et al., 1986; Birnbaumer et al., 1990), it is reasonable to suggest that the soluble multisubunit complex of M_r 98 000 that binds ^{125}I-galanin consists in the soluble galanin receptor component of M_r 57 000 associated to some subunit of a GTP-regulatory protein. To substantiate this assumption, additional experiments were conducted as follows.

Guanine nucleotides known to regulate the binding of galanin to membrane-bound receptors (Lagny-Pourmir et al., 1989a) were tested on the interaction of ^{125}I-galanin to CHAPS-solubilized receptors. Guanine nucleotides, in a dose-dependent manner, dramatically reduced the binding of the tracer to soluble receptors, half-maximal effects being elicited by 2×10^{-5}, 2×10^{-4}, and 5×10^{-4} M for GMP-P(NH)P, GTP, and GDP, respectively (Figure 8). This was due to guanine nucleotide induced dissociation of the soluble galanin–receptor complexes, with 50% dissociation of complexes observed after 30-min incubation in the presence of 10^{-5}

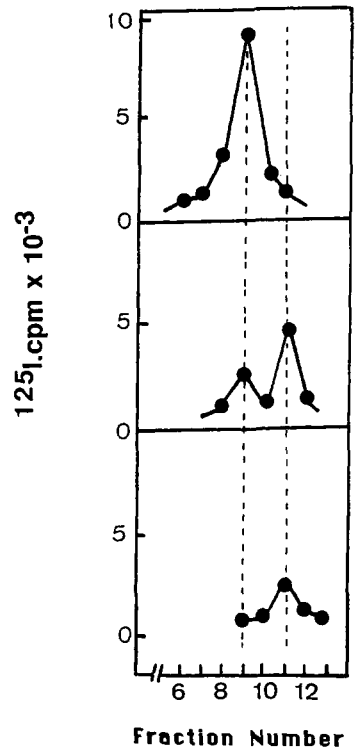

FIGURE 9: Effect of GMP-P(NH)P on the gel filtration profile of solubilized galanin receptors. (Top) CHAPS extracts (2.5 mg of protein/mL) were incubated with ^{125}I-galanin (10 nM) and after elimination of free ^{125}I-galanin (see Materials and Methods) further loaded onto a Sephacryl S-300 column. (Middle) CHAPS extracts were preincubated with 10^{-5} M GMP-P(NH)P for 1 h at 15 °C before incubation with ^{125}I-galanin and further analysis on the Sephacryl S-300 column. (Bottom) CHAPS extracts were preincubated with 10^{-3} M GMP-P(NH)P for 1 h at 15 °C before incubation with ^{125}I-galanin and analysis on the Sephacryl S-300 column.

M GMP-P(NH)P (Figure 2). These observations suggested that the interaction between the galanin receptor and a GTP-regulatory protein survives solubilization of membrane proteins with CHAPS.

To further address this issue, the physical association between the soluble galanin receptor and a GTP-regulatory protein was investigated by studying gel permeation chromatography of soluble galanin receptor in the presence of guanine nucleotides. CHAPS extracts were first preincubated with guanine nucleotides before incubation with ^{125}I-galanin, then sequentially chromatographed on Sephadex G-25 and Sephacryl S-300 columns (see Figure 6), and finally analyzed by ultracentrifugation on a sucrose density gradient (see Figure 7). At 10^{-5} M GMP-P(NH)P (Figure 9, middle), radioactivity in the peak around fraction 9 was decreased as compared to control (Figure 9, top) but a new radioactive peak appeared in fraction 11 of the S-300 column, corresponding to a Stokes radius of 4 nm. When GMP-P(NH)P was used at 10^{-3} M, the initial radioactive peak around fraction 9 totally disappeared while a small radioactive peak in fraction 11 was still observed. This latter radioactive peak did not contain free ^{125}I-galanin that was eliminated by filtration on the Sephadex G-25 column and anyway would be eluted from the Sephacryl S-300 column much later.

The hydrodynamic properties of this new radioactive component were further analyzed to provide an estimate of its molecular weight. For that purpose, the small peak eluted in fraction 11 of the S-300 column was subjected to ultracentrifugation on a linear 5–20% sucrose density gradient. The component of Stokes radius 4 nm migrated with an apparent sedimentation coefficient ($s_{20,w}$) of 3.3 S. The frictional ratio and molecular weight were evaluated at 1.4 and 57 000, respectively (Table I). It is of interest to notice that the molecular weight of this component is identical to the molecular weight of the galanin–receptor complex determined in cross-linking experiments (see Figure 5). Morever, the difference between the molecular weight of the soluble multisubunit complex of 98 000 and the soluble galanin–receptor complex compares well with the molecular weight of α_i subunits of G_i proteins (Birnbaumer et al., 1990) (M_r 41 000) which have been previously shown to couple galanin receptors to adenylate cyclase (Amiranoff et al., 1988). Therefore, it appears that GMP-P(NH)P has allowed the dissociation of such an α subunit of G proteins from the M_r 98 000 complex.

Discussion

In this paper, we demonstrate that the galanin receptor from rat brain membranes can be successfully solubilized in an active and stable state using the zwitterionic detergent CHAPS. In our hands, this detergent was found more efficient than others such as Triton X-100 or digitonin (not shown).

The soluble galanin receptor displays many characteristics similar to those of the membrane form of the receptor. (1) Galanin binds to a single class of soluble receptor with a K_d of 0.8 nM which is similar to the K_d (0.9 nM) for binding to membrane receptor (Servin et al., 1987). Considering the 40% yield of protein solubilization and the binding capacity of soluble extracts (26 fmol/mg of protein), the number of binding sites that are solubilized corresponds to 50% of those measured in rat brain membranes with the same tracer. This indicates that the galanin receptor was not selectively solubilized by CHAPS as compared to total proteins. (2) As observed with membrane receptors (Lagny-Pourmir et al., 1989b), soluble galanin receptors maintain the rank order of affinities for galanin and its fragments, i.e., Gal(1–29) > Gal(2–29) > Gal(1–15), while they did not recognize the 3–29 galanin fragment. (3) The molecular characterization of the galanin receptor binding site by covalent labeling with ^{125}I-galanin in CHAPS extracts and membranes clearly gave rise to similar autoradiographic labeling profiles, indicating that the ligand bound to an identical M_r 54 000 protein in the solubilized and native membranes (see Figure 5).

Gel permeation chromatography of solubilized galanin receptors reveals a protein form with a Stokes radius of 5.03 nm (see Figure 6). This form may represent a single species of galanin receptors but also multiple forms with similar sizes. Prelabeling of receptors in CHAPS extracts or in membranes by ^{125}I-galanin before solubilization gives rise to an identical elution profile, suggesting that occupation of receptors by galanin does not significantly modify the apparent size of soluble receptors. Sucrose density gradient ultracentrifugation of soluble ^{125}I-galanin–receptor complexes also gives evidence for a binding component with a sedimentation coefficient of 4.6 S (see Table I). Since no significant difference is observed between sedimentation coefficient values measured in H_2O and D_2O, it has been assumed that the partial specific volume of galanin receptors is similar to that of globular proteins used as markers (Clarke, 1975). Since the partial specific volume of CHAPS, e.g., 0.81 mL/g (Hones et al., 1986), is different from that of protein markers, e.g., 0.74 mL/g (Clarke, 1975), our data also support that galanin receptors bind a small amount of CHAPS. From the values of Stokes radius and sedimentation coefficient, the molecular weight of the galanin–receptor complex in solution has been estimated at 98 000. This is much higher than the molecular weight of cross-linked ^{125}I-galanin binding site complexes in brain membranes and

CHAPS extracts, e.g., 57 000 (see Figure 5).

Previous studies indicated that galanin inhibition of adenylyl cyclase involves coupling of galanin receptors to a pertussis toxin sensitive G_i protein (Amiranoff et al., 1988). Several arguments do support that the galanin receptor in the soluble state remains functionally and physically associated with the α_i subunit of a G protein. First, galanin receptors are functionally coupled to a G protein in CHAPS extracts since guanine nucleotides inhibit ^{125}I-galanin binding to soluble receptors (see Figure 8). Second, when CHAPS extracts are preincubated with GMP-P(NH)P prior to labeling of receptors with ^{125}I-galanin, the chromatographic profile of the soluble ^{125}I-galanin–receptor complex is modified (see Figure 9). Indeed, the radioactivity in the peak corresponding to a Stokes radius of 5.03 nm, e.g., the M_r 98 000 component (see Table I), is not only decreased, but a second peak of radioactivity is observed, corresponding to a smaller Stokes radius of 4 nm (see Figure 9). After analysis of this new component by sucrose density gradient ultracentrifugation, its molecular weight was estimated at 57 000. These data suggest that guanine nucleotides have induced the dissociation of ^{125}I-galanin but also of α_i subunits from galanin receptors. They also imply that, during GMP-P(NH)P-induced dissociation of α_i from receptors, some ^{125}I-galanin–receptor complexes can survive in our experimental conditions. Third, the molecular weight of the new component generated by GMP-P(NH)P treatment of CHAPS extracts e.g., 57 000, is identical to that determined by affinity cross-linking of galanin–receptor complexes. Morever, the difference in molecular weight of the binding components in the absence, e.g., 98 000, and presence, e.g., 57 000, of guanine nucleotides is 41 000. This is very similar to the molecular weight of α_i subunits in brain and other tissues (Birnbaumer et al., 1990).

Taken together, these data strongly suggested that the galanin receptor remains closely associated with the α subunit of a G_i protein during the membrane solubilization process. Alternatively, they may also indicate the GTP-sensitive association of the galanin receptor with an independent protein or even the GTP-sensitive dimerization of the receptor, two hypotheses that should not be ruled out although they are still not really evidenced. Anyway, as far as the cosolubilization of galanin receptor with a G protein is concerned, this characteristic has been previously reported for other peptide receptors such as liver and intestinal VIP receptors (Couvineau et al., 1990; Calvo et al., 1989) or the pancreatic somatostatin receptor (Knuhtsen et al., 1990). However, it is not a general feature. For instance, CHAPS solubilization of brain neurotensin receptors (Mazella et al., 1988) or gastrin releasing peptide receptor of 3T3 cells (Feldman et al., 1990) was reported to induce the physical disruption of the G-protein coupling to the receptor. Alternatively, the soluble brain CRF receptor is tightly associated with a G protein but not sensitive to guanine nucleotides (Grigoriadis et al., 1989). Whether these discrepancies are related to solubilization conditions or reflect structural specificity in the G-protein–receptor coupling awaits further investigation.

In conclusion, this paper describes the first solubilization and molecular characterization of active receptors for galanin, a neuropeptide widely distributed in mammalian organism. This successful solubilization of functional galanin receptor paves the way for large-scale solubilization and ultimate purification of this recently discovered receptor (Amiranoff et al., 1987).

Registry No. GTP, 86-01-1; CHAPS, 75621-03-3; galanin, 119418-04-1.

References

Amiranoff, B., Servin, A. L., Rouyer-Fessard, C., Couvineau, A., Tatemoto, K., & Laburthe, M. (1987) *Endocrinology 121*, 284–289.

Amiranoff, B., Lorinet, A. M., Lagny-Pourmir, I., & Laburthe, M. (1988) *Eur. J. Biochem. 177*, 147–152.

Amiranoff, B., Lorinet, A. M., & Laburthe, M. (1989a) *J. Biol. Chem. 264*, 20714–20717.

Amiranoff, B., Lorinet, A. M., Yanaihara, N., & Laburthe, M. (1989b) *Eur. J. Pharmacol. 163*, 757–761.

Amiranoff, B., Lorinet, A. M., & Laburthe, M. (1991) *Eur. J. Biochem. 195*, 459–463.

Birnbaumer, L., Abramowitz, J., & Brown, A. M. (1990) *Biochim. Biophys. Acta 1031*, 163–224.

Bradford, M. (1976) *Anal. Biochem. 72*, 248–254.

Bruns, R. F., Lawson-Wendling, K., & Pugsley, T. A. (1983) *Anal. Biochem. 132*, 74–81.

Calvo, J. R., Couvineau, A., Guijarro, L., & Laburthe, M. (1989) *Biochemistry 28*, 1667–1672.

Ch'ng, J. L. C., Christofides, N. D., Anand, P., Gilson, S. J., Allen, Y. S., Su, H. C., Tatemoto, K., Morrison, J. F. B., Polak, J. M., & Bloom, S. R. (1985) *Neuroscience 16*, 343–354.

Clarke, S. (1975) *J. Biol. Chem. 250*, 5459–5469.

Couvineau, A., Amiranoff, B., & Laburthe, M. (1986) *J. Biol. Chem. 261*, 14482–14489.

Couvineau, A., Rouyer-Fessard, C., Voisin, T., & Laburthe, M. (1990) *Eur. J. Biochem. 187*, 605–609.

Ekblad, E., Rökaeus, A., Hakanson, R., & Sundler, F. (1985) *Neuroscience 16*, 355–363.

Feldman, R. I., Wu, J. M., Jenson, J. C., & Mann, E. (1990) *J. Biol. Chem. 265*, 17364–17372.

Fisone, G., Wu, C. F., Consolo, S., Nordstrom, O., Brynne, N., Bartfai, T., Melander, T. & Hokfelt, T. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 7339–7343.

Fisone, G., Langel, U., Carlquist, M., Bergman, T., Consolo, S., Hökfelt, T., Undén, A., Andell, S., & Bartfai, T. (1989a) *Eur. J. Biochem. 181*, 269–276.

Fisone, G., Berthold, M., Bedecs, K., Unden, A., Bartfai, T., Bertorelli, R., Consolo, S., Crauwley, J., Martin, B., Nilsson, S., & Hökfelt, T. (1989b) *Proc. Natl. Acad. Sci. U.S.A. 86*, 9588–9591.

Grigoriadis, D. E., Zaczek, R., Pearsall, D. M., & De Souza, E. B. (1989) *Endocrinology 125*, 3068–3077.

Hone, W. A., Weiland, G. A., & Oswald, R. E. (1986) *J. Biol. Chem. 261*, 3588–3594.

Hunter, W. M., & Greenwood, F. C. (1962) *Nature 194*, 495–496.

Knuhtsen, S., Esteve, J. P., Cambillau, C., Colas, B., Susini, C., & Vaysse, N. (1990) *J. Biol. Chem. 265*, 1129–1133.

Kwok, Y. N., Verchere, C. B., McIntosh, C. H. S., & Brown, J. C. (1988) *Eur. J. Pharmacol. 145*, 49–54.

Laemmli, U. K. (1970) *Nature 227*, 680–685.

Lagny-Pourmir, I., Amiranoff, B., Lorinet, A. M., Tatemoto, K., & Laburthe, M. (1989a) *Endocrinology 124*, 2635–2641.

Lagny-Pourmir, I., Lorinet, A. M., Yanaihara, N., & Laburthe, M. (1989b) *Peptides 10*, 757–761.

Liscia, D. L., Alhadi, T., & Vonderhaar, B. K. (1982) *J. Biol. Chem. 257*, 9401–9405.

Martin, R. G., & Ames, B. W. (1961) *J. Biol. Chem. 154*, 540–552.

Mazella, J., Chabry, J., Kitabgi, P., & Vincent, J. P. (1988) *J. Biol. Chem. 263*, 144–149.

McDonald, T. J., Dupre, J., Tatemoto, K., Greenberg, G. R., Radziuk, J., & Mutt, V. (1985) *Diabetes 34*, 192-196.
Ottlecz, A., Snyder, G. D., & McCann, S. M. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 9861-9865.
Rökaeus, A. (1987) *Trends Neurosci. 10*, 158-164.
Servin, A. L., Amiranoff, B., Rouyer-Fessard, C., Tatemoto, K., & Laburthe, M. (1987) *Biochem. Biophys. Res. Commun. 144*, 298-306.
Silvestre, R. A., Miralles, P., Monge, L., Villanueva, M. L., & Marco, J. (1987) *Life Sci. 40*, 1829-1833.
Tatemoto, K., Rökaeus, A., Jörnvall, H., Mc Donald, T. J., & Mutt, V. (1983) *FEBS Lett. 164*, 124-128.
Voisin, T., Couvineau, A., Rouyer-Fessard, C., & Laburthe, M. (1991) *J. Biol. Chem. 266*, 10762-10767.

Fuc-GM1 Ganglioside Mimics the Receptor Function of GM1 for Cholera Toxin†

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Received May 24, 1991; Revised Manuscript Received December 5, 1991

ABSTRACT: The ability of Fuc-GM1 ganglioside to mimic the receptor function of GM1 for cholera toxin (CT) has been investigated. For this purpose, rat glioma C6 cultured cells were enriched with Fuc-GM1 and the responsiveness to CT was compared with that of cells enriched with GM1 ganglioside. Fuc-GM1 was taken up by cells as rapidly and to the same extent as GM1. When comparable amounts of ganglioside were associated, the cells enriched with Fuc-GM1 bound the same amount of ^{125}I-CT as did cells enriched with GM1. Under conditions in which GM1- and Fuc-GM1-enriched cells bound comparable amounts of CT, the Fuc-GM1-treated cells accumulated virtually the same amount of cyclic AMP as did GM1-treated cells, and activation of adenylate cyclase was also similar. The lag time preceding the CT-induced cAMP accumulation was the same in Fuc-GM1- and GM1-enriched cells. High-sensitivity isothermal titration calorimetry (ITC) experiments showed that the association constants of CT with Fuc-GM1 or GM1 ganglioside were comparable (4×10^7 M^{-1} and 1.9×10^7 M^{-1}, respectively, at 25 °C). Also, the association constants of the B-subunit pentamer with Fuc-GM1 or GM1 ganglioside were comparable (about 3×10^7 M^{-1} and 7×10^7 M^{-1}, respectively, at 25 °C).

Cholera toxin (CT)[1] is composed of two subunits, A and B, the latter being composed of five identical subunits arranged in a pentameric ring. When CT interacts with the host cells it binds through the B-subunit pentamer to its surface receptor. A lag period then follows during which the A subunit penetrates the plasma membrane and splits into peptides A1 and A2; the A1 fragment catalyzes the NAD^+-dependent ADP-ribosylation of the regulatory protein G, which irreversibly binds to, and activates, adenylate cyclase (Holmgren, 1981; Fishman, 1982). GM1 ganglioside has been indicated as the receptor for the toxin (Cuatrecasas, 1973). The ability of GM1 to be taken up by GM1-deficient cells and to sensitize the cells to cholera toxin has further confirmed the ability of GM1 to function as a toxin receptor [for a review about the topic, see Van Heyningen (1983)]. Although other naturally occurring gangliosides have shown the ability to bind CT with high specificity (Nakamura et al., 1987), the cellular responses following this interaction have so far been described only in the case of GM1 ganglioside. In recent years the presence of fucosylated gangliosides, including Fuc-GM1, in normal and transformed cells has been reported and is the focus of increased research (Hakomori, 1989; Chigorno et al., 1982; Ariga et al., 1987). With the present investigation we studied the ability of Fuc-GM1 to function as receptor for CT. The receptor function of Fuc-GM1 has been investigated by testing the ability to elicit cAMP accumulation after functional incorporation into the receptor-deficient rat glioma C6 cells (Fishman, 1980; Fishman et al., 1980) and subsequent exposure to CT, compared with the same cellular system enriched with GM1 ganglioside. The association of CT and its B-subunit pentamer with GM1 and Fuc-GM1 gangliosides has also been directly measured by high-sensitivity isothermal titration calorimetry (ITC), thus providing the overall energetics for the ganglioside–toxin interaction.

Materials and Methods

Reagents and Other Products. Commercial chemicals were of the purest quality available; solvents were distilled and water was doubly distilled in a glass apparatus. Silica gel thin-layer plates (HPTLC, Kieselgel 60) were from Merck (Darmstadt, FRG); solutions for C6 glioma cell culture and washing were from Flow Laboratories (Irwine, U.K.). *N*-Acetylneuraminic acid (NeuAc), bovine serum albumin, cholera toxin, and B-subunit pentamer were purchased from Sigma Chemical Co. (St. Louis, MO). For comparison, CT purified as described by Tomasi et al. (1979), with the phosphocellulose chromatography modification introduced by Mekalanos et al. (1979), was utilized for some experiments and was a kind gift of Dr. Maurizio Tomasi (National Institutes of Health, Rome, Italy).

† Supported by grants from the National Institutes of Health (NS-24520 and RR-04328) and from Consiglio Nazionale delle Ricerche (CNR, Rome, 90.01494.CT04).

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[1] Abbreviations: DMEM, Dulbecco's modification of Eagle's medium; PBS, Dulbecco's phosphate-buffered saline solution without calcium and magnesium; FCS, fetal calf serum; CT, cholera toxin; cAMP, adenosine 3′,5′-(cyclic)monophosphate; DEPC, dielaidoylphosphatidylcholine.

0006-2960/92/0431-2422$03.00/0