0026-895X/93/020167-09$03.00/0

MOLECULAR PHARMACOLOGY, 43:167-175

Solubilization and Characterization of a Guanine Nucleotide-Sensitive Form of the Calcitonin Gene-Related Peptide Receptor

TAPAN K. CHATTERJEE, JADINE A. MOY, JOHN J. CAI, HON-CHI LEE, and RORY A. FISHER

Departments of Pharmacology (T.K.C., J.A.M., R.A.F.) and Internal Medicine (J.J.C., H.-C.L.), The University of Iowa, College of Medicine, Iowa City, Iowa 52242

Received July 20, 1992; Accepted October 22, 1992

SUMMARY

Calcitonin gene-related peptide (CGRP) receptors were solubilized from rat cerebellum membranes in an active, stable, and guanine nucleotide-sensitive form by using digitonin. Nearly 90% of membrane CGRP receptors and 50% of membrane protein were solubilized by digitonin treatment of cerebellum membranes. Binding of ^{125}ICGRP to soluble receptors was specific, saturable, of high affinity, and reversible. Scatchard analysis of the saturation binding data revealed a homogeneous population of binding sites with a K_d of 178 ± 42 pM and a B_{max} of 201 ± 17 fmol/mg of protein. Binding of ^{125}ICGRP to soluble receptors was inhibited nearly 60% by guanosine-5′-*O*-(3-thio)triphosphate (GTPγS) (100 μM), suggesting coupling of receptors with guanine nucleotide-binding proteins (G proteins) to form high affinity binding sites. Antiserum against the amino-terminal region of $G_{s\alpha}$ immunoprecipitated a significant portion of soluble CGRP receptors, indicating association of receptors with $G_{s\alpha}$. In agreement with the saturation binding data, association kinetic studies with soluble receptors indicated binding of ^{125}ICGRP to a single population of sites. Dissociation kinetic data, in contrast, demonstrated that ^{125}ICGRP dissociated from labeled receptors with fast- and slow-dissociating components. GTPγS significantly accelerated dissociation of ^{125}ICGRP from labeled receptors; however, dissociation still occurred from two distinct affinity components, with rate constants significantly different from those observed in the absence of GTPγS. These observations suggest that soluble CGRP receptors, like native membrane-bound receptors, exist in two distinct affinity states in both G protein-coupled and -uncoupled receptor states. Soluble receptors were retained specifically on a wheat germ lectin column, and affinity cross-linking of receptors specifically labeled with ^{125}ICGRP demonstrated labeling of a 67-kDa protein, suggesting that the rat cerebellum CGRP receptor is a 67-kDa glycoprotein. This study is the first to report solubilization of CGRP receptors retaining the native ability of the receptor to undergo functional coupling with G proteins and to provide direct evidence for association of these receptors with $G_{s\alpha}$.

CGRP is a novel neuropeptide produced by tissue-specific alternative splicing of the primary transcript of the calcitonin gene (1, 2). CGRP immunoreactivity is present throughout the central and peripheral nervous system, and release of CGRP from nervous tissue has been demonstrated (2–5). CGRP possesses an impressive array of biological activities consistent with its diverse tissue distribution and its suggested role as a neurotransmitter or neuromodulator. CGRP is located in central cardiovascular control regions and in nerves innervating heart and peripheral resistance vessels and exerts powerful cardiovascular effects including stimulation of cardiac contraction, vasodilation, and central sympathetic outflow (2, 4, 6–11). CGRP is the most potent mammalian vasodilator yet discovered (12). Moreover, CGRP possesses neurotrophic effects and has been implicated in regulation of neuronal differentiation, cell proliferation, nociception, and hormone secretion (13–17). These diverse biological actions of CGRP are thought to result from interactions of CGRP with specific cell receptors in target tissues. Indeed, binding sites for CGRP with properties consistent with those of receptors have been identified in both central and peripheral tissues (3, 18–20).

Stimulation of CGRP receptors in various cells and tissues produces increases in cellular cAMP concentration and/or adenylate cyclase activity (7, 15, 21–23), suggesting that CGRP receptors may couple to G_s and hence belong to the G protein-coupled family of receptors. Analysis of agonist binding to a variety of G protein-coupled receptors has shown that agonists promote or stabilize coupling of receptors to G proteins to form

This work was supported by grants from the National Institutes of Health (HL-41071 and DK-25295), the American Heart Association (891136), and the American Heart Association, Iowa Affiliate (IA-88-G-11). T.K.C. received support from the Roy J. Carver Charitable Trust.

ABBREVIATIONS: CGRP, calcitonin gene-related peptide; G protein, guanine nucleotide-binding protein; G_s, stimulatory guanine nucleotide-binding protein; $G_{s\alpha}$, α subunit of G_s; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; GTPγS, guanosine-5′-*O*-(3-thio)triphosphate); BSA, bovine serum albumin; DSS, disuccinimidyl suberate; DST, disuccinimidyl tartarate; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; VIP, vasoactive intestinal peptide.

an agonist-receptor-G protein ternary complex, a complex that leads to activation of G proteins and intracellular signaling (24, 25). Recently we demonstrated that CGRP receptors in cerebellum and cardiac myocyte membranes exhibit guanine nucleotide-sensitive binding and we provided evidence that the high affinity binding state of CGRP receptors in these tissues results from receptor coupling to G proteins (26, 27). In addition, we found that CGRP receptors exist in more than one affinity state in both G protein-coupled and -uncoupled receptor forms. The functional significance of these multiple affinity states of the CGRP receptor in adenylate cyclase activation and receptor regulation is not known. However, recent studies with adrenergic receptors modified by mutation have raised the intriguing possibility that these receptors may exist in multiple conformational states that differ in their abilities to undergo coupling to G proteins or to activate adenylate cyclase (28, 29). Our results provided the first evidence for the existence of multiple affinity states of a G protein-coupled receptor in its native membrane-bound form.

A prerequisite for further characterization of CGRP receptors and their interactions with G proteins is solubilization of the receptor in a form that retains the intrinsic capability of the receptor to undergo functional coupling with G proteins. We report here that we have achieved nearly complete solubilization of cerebellum CGRP receptors in a form that undergoes functional coupling with G proteins and retains the pharmacological properties of the native membrane-bound receptor.

Experimental Procedures

Materials. Human CGRPα, human calcitonin, and human amylin were purchased from Bachem. Digitonin was purchased from either Sigma Chemical Company or Calbiochem. Fatty acid-free BSA (type V), wheat germ agglutinin-Sepharose 6 MB, and GTPγS were obtained from Sigma. ^{125}ICGRPα (2000 Ci/mmol) was obtained from Amersham. Sephadex G-100 (superfine) was purchased from Pharmacia, and DSS and DST were from Pierce Chemical. Protein standards for SDS-PAGE were obtained from Bio-Rad. All other chemicals used were of analytical grade and of the highest purity available.

Membrane preparation and solubilization. Cerebella were collected from Harlan Sprague-Dawley rats (250 g) of either sex and were homogenized (1:50 wet weight/volume) in 10 mM HEPES, pH 7.4, containing 1 mM EDTA with a Brinkmann Polytron for 20 sec at setting 7. The homogenate was centrifuged at 48,000 × g for 10 min at 4°, and the resulting pellet was washed twice with buffer by rehomogenization and centrifugation. For receptor solubilization, the washed membrane pellet was suspended in 10 mM HEPES, pH 7.4, 1 mM EDTA, 30% glycerol, 3% digitonin, 0.2 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 10 μM leupeptin, 10 μM pepstatin, 10 units/ml aprotinin (buffer A), by brief sonication and the suspension was stirred for 30 min at 4°. Typically, 15 mg of membrane protein were suspended in 5 ml of solubilization buffer, providing a membrane protein/digitonin (w/w) ratio of approximately 1:10. The suspension was then diluted with an equal volume of digitonin-free buffer A and centrifuged at 100,000 × *g* for 60 min. The resulting supernatant was passed through a 0.22-μm filter and used as the soluble receptor preparation. Substitution of other detergents for digitonin in this solubilization procedure was performed to test the efficacy of other detergents for solubilizing cerebellum CGRP receptors. Membranes were always prepared freshly; however, cerebella could be frozen in liquid nitrogen and stored at −80° for up to 1 month before membrane preparation with no differences in ^{125}ICGRP binding. Digitonin-solubilized receptor preparations could be stored at 4° for at least 1 week with no change in ^{125}ICGRP-binding activity.

Binding assay procedures. Standard binding assays were performed at 37° in polypropylene tubes (12 × 75 mm), in a total volume of 100 μl. The assay mixture contained 10 mM HEPES, pH 7.4, 1 mM EDTA, 2 mM $MgCl_2$, 15% glycerol, 0.2% BSA (binding assay buffer), various concentrations of ^{125}ICGRP, and approximately 30 μg of solubilized protein. Binding reactions were performed at 37° for 15 min and thereafter the assay mixture (70 μl) was applied to Sephadex G-100 columns (15 × 0.9 cm; Disposaflex, Kontes, NJ) that were equilibrated at 4° with 10 mM HEPES buffer, pH 7.4, 1 mM EDTA, 2 mM $MgCl_2$, 5% glycerol, 0.05% digitonin. Control experiments showed that specifically bound ^{125}ICGRP did not dissociate from soluble receptors at 4° in the time required (i.e., <10 min) to separate free from bound ^{125}ICGRP on these columns. All ^{125}ICGRP-binding activity eluted from these columns in the first three fractions (1 ml each), which represented the column void volume. In some experiments, free ligand and bound ligand were separated by rapid vacuum filtration over GF/B filters (Whatman) that had been pretreated with polyethyleneimine and BSA, as described previously (26). Specific ^{125}ICGRP binding to soluble receptors was the same using vacuum filtration or G-100 chromatography to separate free and bound ligand; however, nonspecific binding was generally higher using vacuum filtration. Total binding in all assays represented <10% of the ^{125}ICGRP present in the incubation. Nonspecific binding was determined by measurement of ^{125}ICGRP binding in the presence of 0.1 μM unlabeled CGRP. Specific binding represents total binding minus nonspecific binding.

Association and dissociation kinetic studies of ^{125}ICGRP binding to soluble receptors were performed essentially as described previously (26). Dissociation reactions were initiated after equilibrium binding of ^{125}ICGRP (100 pM) to soluble receptors, by addition of unlabeled CGRP to a final concentration of 0.1 μM (volume changes as a result of this addition were <1%).

Chemical cross-linking and SDS-PAGE of solubilized receptor preparations. Soluble receptor preparations (50 μg) were labeled by incubation with ^{125}ICGRP (0.5 nM) under standard binding assay conditions and then separated from free ligand by chromatography on 24 × 1-cm Sephadex G-100 columns. Specific ^{125}ICGRP-binding activity eluted in the void volume of the column. Incubation of column fractions with DSS and DST (0.5 mM each) for 30 min at 4° was performed to chemically cross-link specifically bound ^{125}ICGRP to receptor proteins. The cross-linking reaction was terminated by addition of 50 mM Tris·HCl, pH 7.4. This reaction mixture was applied to a G-100 (15 × 0.9-cm) column and the peak of radioactivity eluting in the column void volume was pooled and subjected to SDS-PAGE (30). Autoradiography of the dried gel was performed at −70° using Kodak XAR film.

Western blotting and immunoprecipitation of $G_{s\alpha}$. Antiserum of designed specificity for $G_{s\alpha}$ was raised to a synthetic peptide corresponding to amino acids 28–42 of $G_{s\alpha}$ (GTP-binding protein fragment of $G_{s\alpha}$; Peninsula Laboratories), as described by Mumby and Gilman (31). Western blotting studies demonstrated that this antiserum, termed $G_{s\alpha}$ antiserum, reacted specifically with $G_{s\alpha}$ and recognized both 45- and 52-kDa forms of $G_{s\alpha}$, as reported previously (32). $G_{s\alpha}$ antiserum was used for Western blotting and for immunoprecipitation of $G_{s\alpha}$ in soluble CGRP receptor preparations. Western blotting of receptor preparations with $G_{s\alpha}$ antiserum was performed essentially as described previously (31), using $G_{s\alpha}$ antiserum at 1/200 dilution and processing blots with ^{125}I-labeled goat antibody to rabbit IgG and autoradiography. For studies investigating immunoprecipitation of $G_{s\alpha}$-CGRP receptor complexes, soluble CGRP receptor preparations (200 μl, ~150 μg of protein) were incubated in buffer B (10 mM HEPES, pH 7.4, 1 mM EDTA, 2 mM $MgCl_2$, 15% glycerol, 1.5% digitonin, 0.2 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 10 μM leupeptin, 10 μM pepstatin, 10 units/ml aprotinin) with 20 μl of undiluted $G_{s\alpha}$ antiserum or preimmune serum for 3 hr at 4°, followed by addition of 20 μl of Protein A-Sepharose and continued incubation at 4° for 1 hr. The samples were then centrifuged at 10,000 × *g* for 2 min to collect supernatant and immunoprecipitate fractions. Immunoprecipitates

were washed twice by resuspension in buffer B and centrifugation and were then resuspended in 300 μl of binding assay buffer. ^{125}ICGRP binding in supernatant and immunoprecipitate fractions was measured using 100 pM ^{125}ICGRP in binding assays and vacuum filtration to separate free and bound ligand, as described above.

Protein was measured according to the method of Bradford (33), using BSA as standard. Binding data were analyzed using the binding programs EBDA, LIGAND, KINETICS, and LOWRY (34). The run test was used to determine the goodness of fit of binding data to a given curve. The *F* test was applied to compare curve fitting for a one- versus two (or more)-binding sites model. Significance of differences between samples was determined by Student's *t* test for comparisons between two groups and by analysis of variance and Sheffe's *post hoc* analysis for multiple comparisons.

Results

A variety of detergents, including CHAPS, cholate, deoxycholate, digitonin, and Triton X-100, were evaluated for their efficacy in solubilizing CGRP receptors from rat cerebellum membranes in a form that was active and stable during incubation at 37°. Digitonin was the most efficacious of the detergents tested and was the only detergent that provided near-quantitative solubilization of ^{125}ICGRP-binding activity from cerebellum membranes. In three separate experiments performed with different cerebellum membrane preparations, digitonin solubilized 87 ± 3% of the ^{125}ICGRP (100 pM)-binding activity and 49 ± 2% of the total protein from rat cerebellum membranes. Inclusion of EDTA and glycerol in the digitonin solubilization buffer was essential for solubilizing CGRP receptors from cerebellum membranes in an active and stable form. In the absence of glycerol, no ^{125}ICGRP-binding activity was detected in the digitonin-solubilized fraction. In the absence of EDTA, solubilized ^{125}ICGRP-binding activity was not stable during incubation at 37° or storage at 4°. Using our solubilization conditions, solubilized preparations of rat cerebellum membranes could be stored for up to 7 days at 4° with no loss of ^{125}ICGRP-binding activity. The binding characteristics of soluble cerebellum CGRP receptors were investigated in incubations performed at 37°.

Fig. 1A illustrates the results of competition binding experiments performed to investigate the specificity of binding of ^{125}ICGRP to soluble cerebellum CGRP receptors. As shown, unlabeled CGRP inhibited binding of ^{125}ICGRP (100 pM) to soluble receptors in a dose-dependent manner, with an IC_{50} of 139 ± 15 pM (five experiments). The slope of the competition binding curve displayed normal steepness with a pseudo-Hill coefficient not significantly different from unity, suggesting that ^{125}ICGRP interacts with a single population of binding sites in the soluble receptor preparation. Concentrations of calcitonin as high as 1 μM produced only minor inhibition of ^{125}ICGRP binding to soluble receptors and amylin, a 37-amino acid peptide with 49% direct sequence homology to CGRP, was >1000-fold less active than CGRP in competing for ^{125}ICGRP-specific binding sites. These observations indicate that binding of ^{125}ICGRP to soluble cerebellum CGRP receptors is highly selective. Scatchard analysis of specific binding of ^{125}ICGRP to solubilized cerebellum CGRP receptors is illustrated in Fig. 1B. As demonstrated by the linearity of the Scatchard plot and by statistical analysis of the saturation binding data by fitting to a one- or two-site binding model using the nonlinear curve-fitting program LIGAND, ^{125}ICGRP interacts with a homogeneous population of binding sites in the soluble receptor preparation with a K_d of 178 ± 42 pM and B_{max} of 201 ± 17 fmol/mg of protein (three experiments). Nonspecific binding of ^{125}ICGRP to soluble receptors increased linearly with increasing ^{125}ICGRP concentration and represented <20% of total binding at 100 pM ^{125}ICGRP, the concentration of ^{125}ICGRP used in our standard binding assays. These results indicate that ^{125}ICGRP binds to a homogeneous population of high affinity binding sites in the soluble receptor preparation.

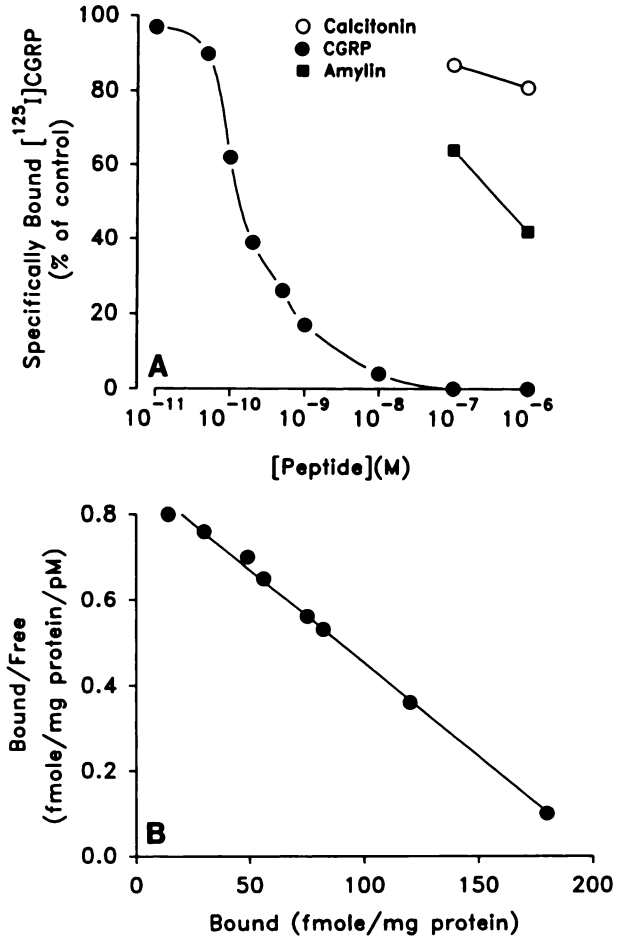

Fig. 1. A, Competition of specific ^{125}ICGRP binding to soluble cerebellum CGRP receptors by CGRP and other peptides. Soluble receptors were incubated with ^{125}ICGRP (100 pM) and varying concentrations of peptides, and the binding assay was performed as described in Experimental Procedures. Data are expressed as percentage of control binding, which refers to the specific binding observed in the absence of competing peptides. Specific binding in control incubations was 55 fmol of ^{125}ICGRP bound/mg of protein, and nonspecific binding was 18% of total binding. B, Scatchard plot of specific binding of ^{125}ICGRP to soluble cerebellum CGRP receptors. Soluble CGRP receptors were incubated with increasing concentrations of ^{125}ICGRP (0.017–1.8 nM) as described in Experimental Procedures. The data represent results from one experiment performed in duplicate and are representative of five (competition) and three (Scatchard) experiments performed with different soluble CGRP receptor preparations.

We demonstrated previously that binding of ^{125}ICGRP to cerebellum membranes is sensitive to guanyl nucleotides, indicating coupling of membrane-bound CGRP receptors to G proteins (26). To investigate whether soluble CGRP receptors retain the ability to undergo coupling with G proteins to form high affinity binding states, binding of ^{125}ICGRP to soluble receptors was examined in the presence and absence of the stable GTP analog GTPγS. As shown in Fig. 2, GTPγS inhib-

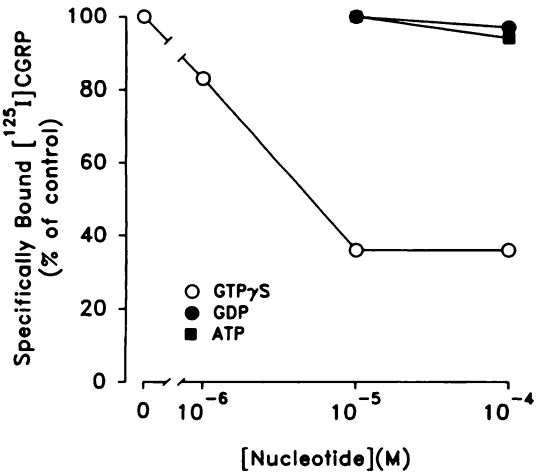

Fig. 2. Inhibition of specific ^{125}ICGRP binding to soluble cerebellum CGRP receptors by GTPγS. Soluble CGRP receptors were incubated, as described in Experimental Procedures, with ^{125}ICGRP (100 pM) and varying concentrations of nucleotides. Data are expressed as percentage of control binding, which refers to specific binding in the absence of nucleotides. Specific binding in control incubations was 58 fmol/mg of protein, and nonspecific binding was 18% of total binding. The data represent results from one experiment performed in duplicate and are representative of four experiments performed with different soluble CGRP receptor preparations.

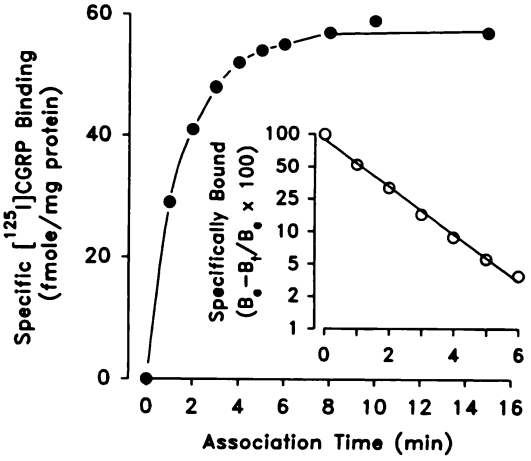

Fig. 3. Association kinetics of specific ^{125}ICGRP (100 pM) binding to soluble cerebellum CGRP receptors. Soluble CGRP receptors were incubated with ^{125}ICGRP (100 pM) for various lengths of time and specific binding of ^{125}ICGRP was assessed as described in Experimental Procedures. *Inset*, pseudo-first-order plot of the association data, where B_e and B_t represent specific binding at equilibrium and at the experimental time point, respectively. The data represent results from one experiment performed in duplicate and are representative of three experiments performed with different soluble CGRP receptor preparations.

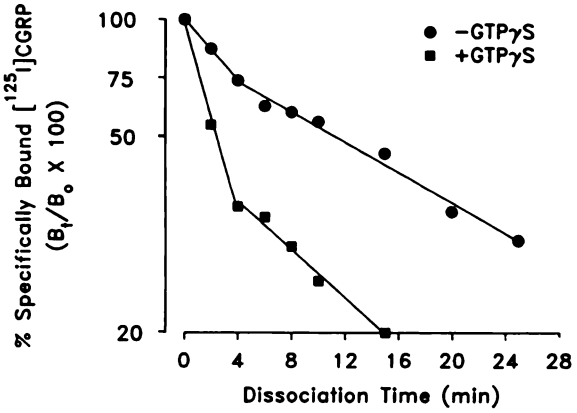

Fig. 4. Effects of GTPγS on dissociation kinetics of specific ^{125}ICGRP binding to soluble cerebellum CGRP receptors. Soluble CGRP receptors were incubated with ^{125}ICGRP (100 pM) for 15 min, in the absence (●) and presence (■) of GTPγS (100 μM). Dissociation was initiated by addition of unlabeled CGRP (0.1 μM), and incubations were terminated at various times thereafter. Specific binding before dissociation (time 0 in the figure) was 62 and 30 fmol of ^{125}ICGRP bound/mg of protein for binding performed in the absence and presence of GTPγS, respectively. The data represent results from one experiment performed in duplicate and are representative of three experiments performed with different soluble CGRP receptor preparations.

ited specific binding of ^{125}ICGRP to soluble receptors in a dose-dependent manner, whereas GDP and ATP were virtually inactive in affecting ^{125}ICGRP binding to soluble receptors. GTPγS inhibited binding of ^{125}ICGRP to soluble CGRP receptors with an IC_{50} of 13 ± 5 μM (four experiments), a value higher than that we observed with the membrane-bound form of the receptor (26). The reason for this decreased sensitivity of soluble CGRP receptors to GTPγS is unclear; however, the extent of maximal inhibition of ^{125}ICGRP binding by GTPγS is comparable between soluble and membrane-bound receptors. In five separate experiments using different preparations of soluble cerebellum CGRP receptors, specific binding of ^{125}ICGRP (100 pM) to soluble receptors in the absence and presence of GTPγS (100 μM) was 53 ± 3 and 24 ± 1 fmol/mg of protein, respectively ($p < 0.01$).

Specific binding of ^{125}ICGRP to soluble cerebellum CGRP receptors was time dependent and reversible. Fig. 3 illustrates the kinetics of association of ^{125}ICGRP (100 pM) with soluble cerebellum CGRP receptors at 37°. As shown, ^{125}ICGRP associated rapidly with soluble receptors and reached binding equilibrium within 8 min. Binding remained stable for at least 45 min after equilibrium binding was attained. The pseudo-first-order association plot of the binding data (Fig. 3, *inset*) was monophasic (with k_{obs} of 0.40 ± 0.07 min^{-1}; three experiments), indicating association of ^{125}ICGRP with a homogeneous population of binding sites. However, the kinetics of dissociation of ^{125}ICGRP from receptors labeled to equilibrium with ^{125}ICGRP (100 pM) were curvilinear, indicating dissociation of ^{125}ICGRP from binding sites of more than one affinity (Fig. 4). The dissociation data fit best to a model of dissociation from two affinity states in which the fast- and slow-dissociating receptor states have dissociation rate constants of 0.47 ± 0.06 and 0.024 ± 0.005 min^{-1} (six experiments), respectively, with the fast-dissociating component representing 37 ± 7% of the ^{125}ICGRP-labeled sites. In view of these findings and the observation that soluble cerebellum CGRP receptors display GTPγS-sensitive binding (Fig. 2), we investigated the possibility that the two rates of dissociation of ^{125}ICGRP from solubilized receptors represent dissociation from G protein-coupled and -uncoupled receptors. To consider this possibility, soluble CGRP receptors were labeled with ^{125}ICGRP in the presence of GTPγS (100 μM) to prevent formation of G protein-coupled receptor states. Then, dissociation was initiated in the continued presence of GTPγS. As shown in Fig. 4, dissociation of ^{125}ICGRP from soluble receptors occurred much more rapidly under these conditions. However, the kinetics of dissociation remained curvilinear, with the dissociation data fitting best to a model of dissociation from two affinity states with dissociation rate constants of 0.79 ± 0.09 and 0.069 ± 0.016 min^{-1} (three exper-

iments). The fast-dissociating (low affinity) component represented 58 ± 2% of the sites occupied by ^{125}ICGRP under these labeling and dissociation conditions. The dissociation rate constants for the high and low affinity ^{125}ICGRP binding sites observed in the presence of GTPγS were significantly greater ($p < 0.05$) than those observed in the absence of GTPγS. In addition, ^{125}ICGRP binding sites with similar dissociation rate constants were not observed under these two experimental conditions.

The sensitivity of the ligand-binding activity of soluble CGRP receptors to GTPγS indicates coupling of the soluble receptor to a G protein(s). In nearly all tissues examined, CGRP stimulates increases in adenylate cyclase activity and/or cAMP concentration, suggesting coupling of CGRP receptors to G_s. However, direct evidence for coupling of CGRP receptors to G_s has not been reported. To examine whether G_s was present in soluble CGRP receptor preparations, Western analysis of the receptor preparation with antiserum against a peptide corresponding to amino acids 28–42 of $G_{s\alpha}$ was performed. As shown in Fig. 5, immunoblotting with $G_{s\alpha}$ antiserum demonstrated the presence of the 45-kDa $G_{s\alpha}$ subunit in cerebellum membranes (Fig. 5, *lane 2*) and the soluble receptor preparation (Fig. 5, *lane 3*), with more $G_{s\alpha}$ immunoreactivity being present in the soluble receptor preparation. Because equal amounts of protein (25 μg) were loaded in each of these lanes, the apparent enrichment of 45-kDa $G_{s\alpha}$ immunoreactivity in the soluble receptor preparation, compared with membranes, may reflect selective solubilization of $G_{s\alpha}$ over other membrane proteins, i.e., as we observed for solubilization of CGRP receptors from these membranes (see above). As reported previously by Mumby *et al.* (32), this antiserum detected both 45- and 52-kDa $G_{s\alpha}$ subunits in cardiac sarcolemma (Fig. 5, *lane 1*) and did not react with proteins with subunit molecular weights corresponding to those of other known $G_{s\alpha}$ subunits (i.e., 36,000–41,000) in any of the samples tested. We next studied CGRP receptor-$G_{s\alpha}$ association by investigating whether CGRP receptor-G_s complexes could be immunoprecipitated with peptide-directed antiserum against $G_{s\alpha}$. Fig. 6 shows that $G_{s\alpha}$ antiserum immunoprecipitated significant specific ^{125}ICGRP-binding activity from soluble CGRP receptor preparations. This is shown as a significant increase in specific ^{125}ICGRP-binding activity in $G_{s\alpha}$ antiserum immunoprecipitates, compared with preimmune serum controls, with a corresponding decrease in binding activity in supernatants after immunoprecipitation with $G_{s\alpha}$ antiserum. The loss of specific ^{125}ICGRP-binding activity from supernatants of $G_{s\alpha}$ antiserum immunoprecipitates represented 35 ± 2% ($p < 0.01$; four experiments) of the ^{125}ICGRP-binding activity of the soluble receptor preparation, compared with preimmune serum controls. The finding that treatment of soluble receptors with $G_{s\alpha}$ antiserum caused a shift in ^{125}ICGRP-binding activity from the supernatant to the immunoprecipitate fraction without decreasing total binding activity suggests that $G_{s\alpha}$ antiserum immunoprecipitates CGRP receptor-G_s complexes and does not uncouple CGRP receptors from G_s or other G proteins.

Fig. 7 illustrates the results of experiments performed to determine the molecular weight of soluble CGRP receptors by affinity cross-linking. Soluble CGRP receptors migrated on sucrose gradients as a discrete peak of specific ^{125}ICGRP-binding activity with a sedimentation coefficient of 14.5 S (data not shown); however, estimates of the molecular weight of digitonin-solubilized proteins by hydrodynamic methods are confounded by the fact that the partial specific volume of digitonin is nearly the same as that of most proteins. For affinity cross-linking studies, soluble receptors were first labeled with 500 pM ^{125}ICGRP and then separated from unbound ligand by Sephadex G-100 chromatography. Fig. 7A shows that

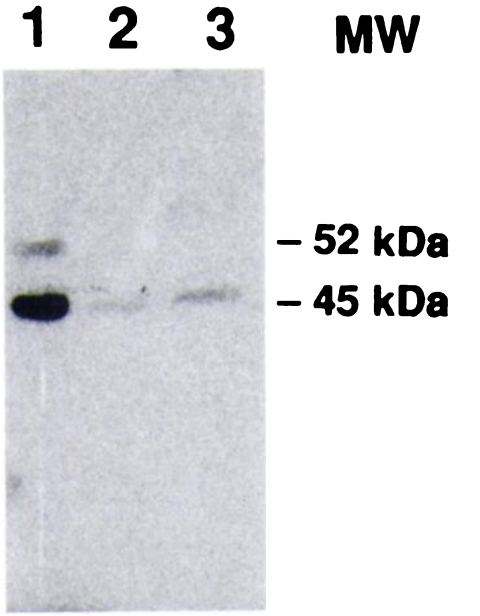

Fig. 5. Presence of $G_{s\alpha}$ immunoreactivity in soluble cerebellum CGRP receptor preparations. Equivalent amounts of protein (25 μg) from cardiac sarcolemma membranes (*lane 1*), rat cerebellum membranes (*lane 2*), and soluble rat cerebellum CGRP receptor preparations (*lane 3*) were subjected to SDS-PAGE and Western blotting using $G_{s\alpha}$ antiserum (1/200). Blots were processed with ^{125}I-labeled goat antibody to rabbit IgG followed by autoradiography, as described in Experimental Procedures. The figure shows a typical autoradiogram from such an experiment and is representative of results obtained in three other experiments performed with different cerebellum membrane and soluble cerebellum receptor preparations. *Numbers to the right*, apparent molecular masses of immunoreactive bands.

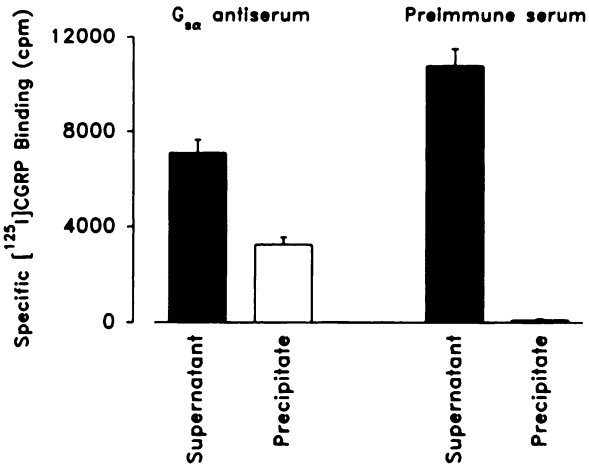

Fig. 6. Immunoprecipitation of soluble cerebellum CGRP receptors with $G_{s\alpha}$ antiserum. Soluble CGRP receptors were incubated with $G_{s\alpha}$ antiserum or preimmune serum and immunoprecipitated as described in Experimental Procedures. The presence of CGRP receptors in supernatant and immunoprecipitate fractions was assessed by specific ^{125}ICGRP (100 pM) binding assays. The values shown represent means ± standard errors of specific ^{125}ICGRP-binding activity in supernatant and immunoprecipitate fractions from four separate experiments, each performed in duplicate.

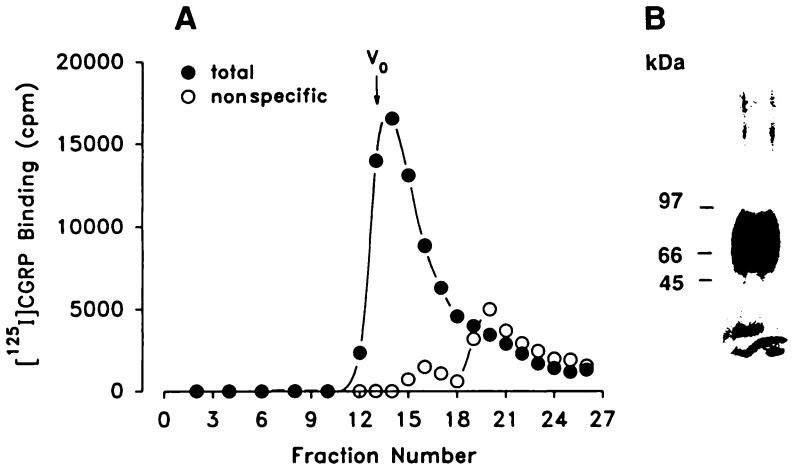

Fig. 7. Sephadex G-100 chromatography and affinity cross-linking of ^{125}ICGRP-labeled soluble cerebellum CGRP receptors. A, Sephadex G-100 chromatography of soluble CGRP receptors. Soluble receptors were first labeled by incubation with ^{125}ICGRP (0.5 nM) in the absence (●) (total binding) and presence (○) (nonspecific binding) of unlabeled CGRP (0.1 μM) and were then subjected to Sephadex G-100 chromatography as described in Experimental Procedures. V_0, column void volume, as determined by elution of blue dextran. The three peak fractions of ^{125}ICGRP-binding activity (fractions 13–15), in which specific ^{125}ICGRP binding was >98% of total binding, were treated with DSS and DST to chemically cross-link specifically bound ^{125}ICGRP to CGRP receptors. B, Autoradiogram of affinity cross-linked receptors after SDS-PAGE analysis.

the specific ^{125}ICGRP-binding activity of the soluble receptor preparation eluted in the void volume of the column. This is shown by the near-complete inhibition of ^{125}ICGRP-binding activity in the void volume of the column when labeling was performed in the presence of 0.1 μM CGRP (nonspecific binding). The three peak fractions of ^{125}ICGRP-binding activity, in which specific binding represented >98% of total ^{125}ICGRP-binding activity, were incubated with DSS and DST to chemically cross-link the specifically bound ^{125}ICGRP to receptors. SDS-PAGE analysis of specifically labeled receptors revealed a band of radioreactivity centered at an apparent molecular mass of 71 kDa (Fig. 7, *inset*). Labeling of the 71-kDa band was observed in all receptors preparations tested (seven experiments), although the broadness of the band varied from 2–3 kDa to 4–8 kDa with different receptor preparations and exposure times. The autoradiogram shown in Fig. 7B was exposed to illustrate the absence of other radiolabeled bands in the soluble receptor preparation.

Many receptors, including G protein-coupled receptors, are glycosylated and bind to various lectins. Fig. 8 illustrates elution of specific ^{125}ICGRP-binding activity from a wheat germ agglutinin-Sepharose column to which soluble receptors had been absorbed by incubation at 4° for 4 hr. As shown, a large peak of specific ^{125}ICGRP-binding activity was eluted from the column by addition of *N*-acetylglucosamine (0.25 M) to the elution buffer. These results demonstrate the glycoprotein nature of cerebellum CGRP receptors. However, as observed with other glycoprotein receptors (35, 36), some CGRP receptors (54 ± 2%; three experiments) were not retained on the column using these absorption and elution conditions.

Discussion

The results of the present study document the successful solubilization of an active, stable, guanine nucleotide-sensitive form of the cerebellum CGRP receptor that retains the binding characteristics of the native membrane-bound form of the receptor. The soluble receptor preparation exhibited high affinity ^{125}ICGRP-binding activity that was saturable and selective.

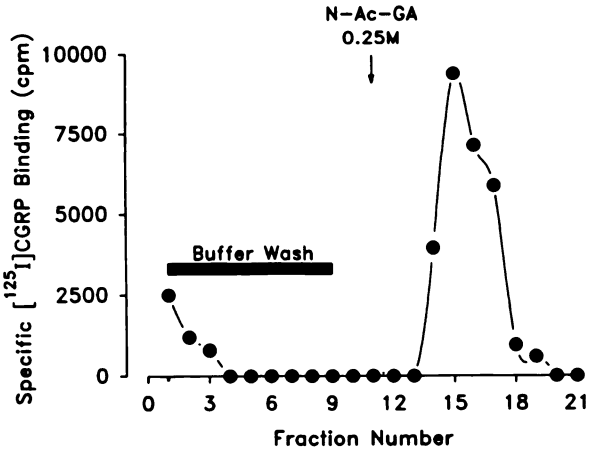

Fig. 8. Elution profile of soluble cerebellum CGRP receptors from a wheat germ lectin affinity column. Soluble CGRP receptors (0.5 mg of protein) were incubated for 4 hr at 4° with 5 ml (packed resin) of wheat germ agglutinin-Sepharose 6 MB in a column equilibrated in 10 mM HEPES, pH 7.4, 1 mM EDTA, 2 mM $MgCl_2$, 5% glycerol, 0.05% digitonin. The column was drained and then washed with equilibration buffer, with fractions (0.5 ml) being collected during washing. The column was then specifically eluted with equilibration buffer containing 0.25 M *N*-acetylglucosamine (*N-Ac-GA*). *Arrow*, point where elution was started with *N*-acetylglucosamine-containing buffer. All fractions were assayed for specific ^{125}ICGRP binding as described in Experimental Procedures.

Our results demonstrate that the binding activity of soluble CGRP receptors is sensitive to guanine nucleotides and that these receptors are capable of direct physical interaction with the α subunit of G_s. This study is the first to report solubilization of a guanine nucleotide-sensitive form of the CGRP receptor and to provide direct evidence for coupling of these receptors to G_s. The present findings also suggest that soluble CGRP receptors, like their membrane-bound counterparts, exist in multiple affinity binding states. Moreover, the data presented here provide biochemical evidence that the CGRP receptor is a glycoprotein with a subunit molecular mass of approximately 67 kDa.

The ligand-binding properties of soluble CGRP receptors

described in the present study are nearly indistinguishable from those of the membrane-bound receptor that we described recently (26). Our solubilization procedures were developed specifically to solubilize CGRP receptors from cerebellum membranes quantitatively and in a form remaining active at 37°. This enabled us to examine the binding characteristics of the soluble receptor at physiological temperatures and to make direct comparisons with the properties of the receptor in its membrane-bound state. Although solubilization of CGRP receptors has been reported (37–39), the present study is the first to undertake a detailed examination of the binding properties of soluble CGRP receptors and to perform these studies at 37°. As we observed for CGRP receptors in cerebellum membranes, binding of ^{125}ICGRP to soluble CGRP receptors was saturable, of high affinity, highly selective, and sensitive to guanine nucleotides. The Scatchard plot of ^{125}ICGRP binding to soluble receptors was linear, indicating a homogeneous population of binding sites with a K_d of 178 ± 42 pM. Thus, the affinity of soluble CGRP receptors for ^{125}ICGRP is equivalent to that of receptors in the membrane-bound state (i.e., 224 ± 28 pM) (26). Our results indicate that the high affinity binding state of soluble CGRP receptors results from receptor coupling to G proteins. Indeed, ^{125}ICGRP binding to soluble receptors was inhibited specifically by GTPγS, and rates of dissociation of ^{125}ICGRP from receptors labeled in the presence of GTPγS were increased significantly, compared with controls, reflecting decreased receptor affinity for CGRP after uncoupling of receptors from G proteins (24, 25). These binding experiments demonstrate for the first time that soluble CGRP receptors are functionally associated with G protein(s).

The data presented here suggest that soluble CGRP receptors exist as a homogeneous population of receptors that undergo ligand-induced formation of multiple affinity binding states in both G protein-coupled and -uncoupled receptor forms. In agreement with saturation binding data indicating a single class of ^{125}ICGRP binding sites in soluble CGRP receptors, association of ^{125}ICGRP with receptors exhibited monoexponential kinetics. In contrast, dissociation of ^{125}ICGRP from labeled receptors displayed multiexponential kinetics best described by dissociation of the ligand from two distinct affinity states of the receptor. Although multiexponential dissociation could result from CGRP receptor heterogeneity, the linear Scatchard plot and association kinetic data argue against this possibility, as follows. If multiexponential dissociation of ^{125}ICGRP from soluble receptors results from binding of ^{125}ICGRP to receptor subtypes with distinct kinetic and equilibrium binding characteristics, we would expect to observe curvilinear association and/or Scatchard plots of ^{125}ICGRP binding to these receptors. If such receptor subtypes exist, the observed monophasic association plot of ^{125}ICGRP binding to soluble receptors indicates that the receptor subtypes have the same association rate constant for ^{125}ICGRP binding. However, ^{125}ICGRP dissociated from receptors with two distinct rates, with dissociation rate constants that differed by >10-fold. Thus, if distinct receptor subtypes account for the multiexponential dissociation reaction, then these subtypes would exhibit K_d values that differ by a minimum of 10-fold (i.e., $K_d = k_{dissociation}/k_{association}$). This difference, which would be readily apparent in Scatchard analysis of the saturation binding data, was not observed. Thus, our data are not at all consistent with CGRP receptor heterogeneity. As we discussed in detail previously (26), such results are entirely consistent with a multistep binding reaction process in which initial binding of CGRP to a homogeneous population of binding sites induces the formation of multiple affinity states of the receptor. In such a case the Scatchard plot of the saturation binding data is linear because the binding equilibria are interdependent in the multistep binding reaction and the K_d of the overall binding reaction is equal to the product of the equilibrium constants of the individual steps. Multiple affinity states of the receptor could result either from receptor isomerization or from receptor coupling to G protein(s), with the latter resulting in an equilibrium between G protein-coupled (high affinity, slow-dissociating) and -uncoupled (low affinity, fast-dissociating) forms of the receptor. In the latter case, GTPγS would be expected to shift this equilibrium to the low affinity state by preventing formation of the ligand-receptor-G protein ternary complex. In fact, GTPγS significantly accelerated dissociation of ^{125}ICGRP from soluble receptors. However, dissociation of ^{125}ICGRP from soluble receptors in the presence of GTPγS still occurred with two distinct components of dissociation, with rate constants significantly different from those observed in the absence of GTPγS. Thus, the two rates of dissociation of ^{125}ICGRP from soluble receptors are both sensitive to GTPγS, indicating the existence of two distinct affinity states of the ligand-receptor-G protein ternary complex. The finding that two different components of dissociation were observed also with receptors incubated in the presence of GTPγS indicates that soluble CGRP receptors also exist in two distinct affinity states when uncoupled from G proteins.

The functional significance of multiple affinity forms of the CGRP receptor is not known. Our previous studies with the membrane-bound form of this receptor provided initial experimental evidence that G protein-coupled receptors may exist in more than one affinity state in both G protein-coupled and -uncoupled receptor forms (26, 27). Recent mutational analysis of adrenergic receptors has provided evidence for the possible existence of more than one G protein-coupled and -uncoupled form of these receptors, which differ in their signal transduction activities (28, 29). Thus, it is interesting to speculate that multiple affinity states of the CGRP receptor may represent unique functional states of the receptor; however, this possibility remains to be investigated.

Our ability to immunoprecipitate soluble CGRP receptors with peptide antiserum directed against $G_{s\alpha}$ provides the first direct evidence for coupling of CGRP receptors to $G_{s\alpha}$. We considered that CGRP receptors might physically couple to $G_{s\alpha}$, for several reasons. First, previous studies have shown that CGRP increases cAMP concentration and/or adenylate cyclase activity in various tissues (7, 15, 21–23). Second, our binding studies indicate that CGRP receptors couple with endogenous G proteins in the soluble extract to form high affinity binding states. Third, numerous studies have shown that coupling of soluble receptors to G proteins involves physical association between these proteins, resulting in formation of receptor-G protein complexes (40–45). Several recent studies have identified the species of G protein coupled to receptors by immunoprecipitation of receptor-G protein complexes with peptide-directed antisera against G protein α subunits (44, 45). We employed a similar approach to detect coupling of CGRP receptors to G_s, using a synthetic peptide antiserum directed against the amino-terminal region of $G_{s\alpha}$, i.e., a region present in both 45- and 52-kDa forms of $G_{s\alpha}$ that is not thought to be

involved in coupling to receptors (46, 47). Our results show that a significant proportion of soluble CGRP receptors were immunoprecipitated with antiserum against $G_{s\alpha}$. Immunoprecipitation of CGRP receptors was achieved in the absence of prebound agonist ligand, suggesting that soluble CGRP receptors exist precoupled to $G_{s\alpha}$. Similar findings were reported by Law *et al.* (45), who demonstrated that immunoprecipitation of soluble somatostatin receptors with antisera to α subunits of G_i did not require agonist pretreatment of receptors. This property of precoupling of receptors to G proteins has been reported previously for other receptors (43, 48, 49); however, it appears that some receptors require the presence of agonist during their solubilization to induce coupling to G proteins (40–42, 44). The extent of immunoprecipitation of CGRP receptors with $G_{s\alpha}$ antiserum observed in the present study is comparable to that reported recently for immunoprecipitation of muscarinic and somatostatin receptors with G protein-directed antisera (44, 45). It is interesting to speculate that CGRP receptors may be coupled predominantly to the 45-kDa form of $G_{s\alpha}$, because Western analysis of the soluble receptor preparation demonstrated a single immunoreactive band at 45 kDa.

Recently, Kermode *et al.* (50) provided evidence for coupling of VIP receptors to $G_{s\alpha}$, using a novel cross-linking approach. Chemical cross-linking of VIP receptors with ^{125}IVIP labeled a single protein of 57 kDa. Subjecting the ^{125}IVIP-receptor complex to a second round of cross-linking with different cross-linking agents resulted in formation of higher molecular weight radiolabeled bands that displayed immunoreactivity to $G_{s\alpha}$ antiserum and that were not observed in the presence of GTP. These data were interpreted as evidence for functional coupling of VIP receptors to $G_{s\alpha}$ and were offered as an explanation for the diversity of reported molecular weight estimates for this receptor by chemical cross-linking studies. Thus, it is of interest to note that previous cross-linking studies of CGRP receptors in various tissues have demonstrated labeling of proteins ranging from 17 to 240 kDa, with more than one specifically labeled band being observed in a single tissue (37–39). Moreover, the hypothesis has been put forth that cross-linking of 70- and 120-kDa proteins in heart with ^{125}ICGRP may represent G protein-uncoupled and -coupled forms of the CGRP receptor, respectively, although no evidence for this possibility was presented (38).

It was with these observations in mind that we investigated the subunit molecular weight of soluble CGRP receptors by prelabeling receptors with ^{125}ICGRP and isolating specifically labeled receptors by G-100 chromatography. It is clear from the present study that such receptors exist exclusively in a ligand-receptor-G protein complex, as shown by dissociation kinetic experiments. When these receptors were cross-linked and analyzed by SDS-PAGE and autoradiography, we observed labeling of a single 71-kDa band, leading us to deduce a subunit molecular mass of the CGRP receptor of 67 kDa. No evidence for labeling of proteins of higher or lower subunit molecular mass was apparent. This estimate of the molecular mass of the CGRP receptor is in the range reported for other G protein-coupled receptors. The broadness of the ^{125}ICGRP-labeled band observed in this study has been observed with other G protein-coupled receptors and has been attributed to heterogeneity in carbohydrate residues on the protein. This possibility seems likely, in part because of our finding that only 54 ± 2% (three experiments) of soluble CGRP receptors labeled with ^{125}ICGRP were retained on a wheat germ lectin column. Finally, it is interesting to note that the observed elution of soluble receptors in the void volume of Sephadex G-100 columns, indicating an apparent molecular mass of >100 kDa, is consistent with the expected elution of a receptor of 67 kDa complexed to a G protein. Similar findings have been reported for other G protein-coupled receptors, although we acknowledge that our findings could also result from association of the detergent-solubilized receptor with other proteins or from anomalous chromatographic behavior.

Further work will be required to define the molecular basis of interactions between CGRP, CGRP receptors, and G proteins that lead to receptor activation, multiple affinity states of the receptor, and signal transduction. The capability of solubilizing CGRP receptors in a form retaining the native characteristics of the membrane-bound receptor, including association and functional coupling with G proteins, hopefully will lead to a more complete molecular and functional understanding of this neuropeptide receptor.

References

1. Amara, S. G., V. Jonas, M. G. Rosenfeld, E. S. Ong, and R. M. Evans. Alternative RNA processing in calcitonin gene expression generates mRNAs encoding different polypeptide products. *Nature* (*Lond.*) **298**:240–244 (1982).
2. Rosenfeld, M. G., J. J. Mermod, S. G. Amara, L. W. Swanson, J. Rivier, W. W. Vale, and R. M. Evans. Production of a novel neuropeptide encoded by the calcitonin gene via tissue-specific RNA processing. *Nature* (*Lond.*) **304**:129–135 (1983).
3. Kruger, L., P. W. Mantyh, C. Sternini, N. C. Brecha, and C. R. Mantyh. Calcitonin gene-related peptide (CGRP) in the rat central nervous system: patterns of immunoreactivity and receptor binding sites. *Brain Res.* **463**:223–244 (1988).
4. Mulderry, P. K., M. A. Ghatei, J. Rodrigo, J. M. Allen, M. G. Rosenfeld, J. M. Polak, and S. R. Bloom. Calcitonin gene-related peptide in cardiovascular tissues of the rat. *Neuroscience* **14**:947–954 (1985).
5. Mason, R. T., R. A. Peterfreund, P. E. Sawchenko, A. Z. Corrigan, J. E. Rivier, and W. W. Vale. Release of the predicted calcitonin gene-related peptide from cultured rat trigeminal ganglion cells. *Nature* (*Lond.*) **308**:653–655 (1984).
6. Goodman, E. C., and L. L. Iversen. Calcitonin gene-related peptide: novel neuropeptide. *Life Sci.* **39**:2169–2178 (1986).
7. Sigrist, S., A. Franco-Cereceda, R. Muff, H. Henke, J. M. Lundberg, and J. A. Fischer. Specific receptor and cardiovascular effects of human calcitonin gene-related peptide. *Endocrinology* **119**:381–389 (1986).
8. Saito, A., S. Kimura, and K. Goto. Calcitonin gene-related peptide as potential neurotransmitter in guinea pig right atrium. *Am. J. Physiol.* **250**:H693–H698 (1986).
9. Lundberg, J. M., A. Franco-Cereceda, X. Hua, T. Hokfelt, and J. A. Fischer. Co-existence of substance P and calcitonin gene-related peptide-like immunoreactivities in sensory nerves in relation to cardiovascular and bronchoconstrictor effects of capsacin. *Eur. J. Pharmacol.* **108**:315–319 (1985).
10. Franco-Cereceda, A., and J. M. Lundberg. Calcitonin gene-related peptide (CGRP) and capsaicin-induced stimulation of heart contractile rate and force. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **331**:146–151 (1985).
11. Fisher, L. A., D. O. Kikkawa, J. E. Rivier, S. G. Amara, R. M. Evans, M. G. Rosenfeld, W. W. Vale, and M. R. Brown. Stimulation of noradrenergic sympathetic outflow by calcitonin gene-related peptide. *Nature* (*Lond.*) **305**:534–536 (1983).
12. Brain, S. D., T. J. Williams, J. R. Tippins, H. R. Morris, and I. MacIntyre. Calcitonin gene-related peptide is a potent vasodilator. *Nature* (*Lond.*) **313**:54–56 (1985).
13. New, H. V., and A. W. Mudge. Calcitonin gene-related peptide regulates muscle acetylcholine receptor synthesis. *Nature* (*Lond.*) **323**:809–811 (1986).
14. Denis-Donini, S. Expression of dopaminergic phenotypes in the mouse olfactory bulb induced by the calcitonin gene-related peptide. *Nature* (*Lond.*) **339**:701–703 (1989).
15. Haegerstrand, A., C.-J. Dalsgaard, B. Jonzon, O. Larsson, and J. Nilsson. Calcitonin gene-related peptide stimulates proliferation of human endothelial cells. *Proc. Natl. Acad. Sci. USA* **87**:3299–3303 (1990).
16. Woolf, C., and Z. Wiesenfeld-Hallin. Substance P and calcitonin gene-related peptide synergistically modulate the gain of the nociceptive flexor withdrawal reflex in the rat. *Neurosci. Lett.* **66**:226–230 (1986).
17. Pettersson, M., B. Ahren, G. Bottcher, and F. Sundler. Calcitonin gene-related peptide: occurrence in pancreatic islets in the mouse and the rat and the inhibition of insulin secretion in the mouse. *Endocrinology* **119**:865–869 (1986).
18. Skofitsch, G., and D. M. Jacobowitz. Autoradiographic distribution of ^{125}I-

calcitonin gene-related peptide binding sites in the rat central nervous system. *Peptides* **4**:975–986 (1985).
19. Tschopp, F. A., H. Henke, J. B. Peterman, P. H. Tobler, R. Janzer, T. Hokfelt, J. M. Lundberg, C. Cuello, and J. A. Fischer. Calcitonin gene-related peptide and its binding sites in the human nervous system and pituitary. *Proc. Natl. Acad. Sci. USA* **82**:248–252 (1985).
20. Goltzman, D., and J. Mitchell. Interaction of calcitonin and calcitonin gene-related peptide at receptor sites in target tissues. *Science (Washington D. C.)* **227**:1343–1345 (1985).
21. Kubota, M., J. M. Moseley, L. Butera, G. J. Dusting, P. S. MacDonald, and T. J. Martin. Calcitonin gene-related peptide stimulates cyclic AMP formation in rat aortic smooth muscle cells. *Biochem. Biophys. Res. Commun.* **132**:88–94 (1985).
22. Yamaguchi, A., T. Chiba, T. Yamatani, T. Inui, T. Morishita, A. Nakamura, S. Kadowaki, M. Fukase, and T. Fujita. Calcitonin gene-related peptide stimulates adenylate cyclase activation via a guanine nucleotide-dependent process in rat liver plasma membranes. *Endocrinology* **123**:2591–2596 (1988).
23. Fisher, R. A., S. M. Robertson, and M. S. Olson. Stimulation and homologous desensitization of calcitonin gene-related peptide receptors in cultured beating rat heart cells. *Endocrinology* **123**:106–112 (1988).
24. De Lean, A., J. M. Stadel, and R. J. Lefkowitz. A ternary complex model explains the agonist-specific binding properties of the adenylate cyclase-coupled β-adrenergic receptor. *J. Biol. Chem.* **255**:7108–7117 (1980).
25. Birnbaumer, L., J. Abramowitz, and A. M. Brown. Receptor-effector coupling by G proteins. *Biochim. Biophys. Acta* **1031**:163–224 (1990).
26. Chatterjee, T. K., and R. A. Fisher. Multiple affinity forms of the calcitonin gene-related peptide receptor in rat cerebellum. *Mol. Pharmacol.* **39**:798–804 (1991).
27. Chatterjee, T. K., J. A. Moy, and R. A. Fisher. Characterization and regulation of high affinity calcitonin gene-related peptide receptors in cultured neonatal rat cardiac myocytes. *Endocrinology* **128**:2731–2738 (1991).
28. Hausdorff, W. P., M. Hnatowich, B. F. O'Dowd, M. G. Caron, and R. J. Lefkowitz. A mutation of the β_2-adrenergic receptor impairs agonist activation of adenylyl cyclase without affecting high affinity agonist binding. *J. Biol. Chem.* **265**:1388–1393 (1990).
29. Kjelsberg, M. A., S. Cotecchia, J. Ostrowski, M. G. Caron, and R. J. Lefkowitz. Constitutive activation of the α_{1B}-adrenergic receptor by all amino acid substitutions at a single site. *J. Biol. Chem.* **267**:1430–1433 (1992).
30. Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. *Nature (Lond.)* **227**:680–685 (1970).
31. Mumby, S. M., and A. G. Gilman. Synthetic peptide antisera with determined specificity for G protein α or β subunits. *Methods Enzymol.* **195**:215–233 (1991).
32. Mumby, S. M., R. A. Kahn, D. R. Manning, and A. G. Gilman. Antisera of designed specificity for subunits of guanine nucleotide-binding regulatory proteins. *Proc. Natl. Acad. Sci. USA* **83**:265–269 (1986).
33. Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**:248–254 (1976).
34. McPhearson, G. A. *KINETIC, EBDA, LIGAND, LOWRY: A Collection of Radioligand Binding Analysis Programs.* Elsevier Science Publishers BV, Amsterdam, The Netherlands (1985).
35. Stiles, G. L., J. L. Benovic, M. G. Caron, and R. J. Lefkowitz. Mammalian β-adrenergic receptors. *J. Biol. Chem.* **259**:8655–8663 (1984).
36. Voisin, T., A. Couvineau, C. Rouyer-Fessard, and M. Laburte. Solubilization and hydrodynamic properties of active peptide YY receptor from rat jejunal crypts. *J. Biol. Chem.* **266**:10762–10767 (1991).
37. Foord, S. M., and R. K. Craig. Isolation and characterization of a human calcitonin gene-related-peptide receptor. *Eur. J. Biochem.* **170**:373–379 (1987).
38. Sano, Y., O. Hiroshima, T. Yuzuriha, C. Yamato, A. Saito, S. Kimura, T. Hirabayashi, and K. Goto. Calcitonin gene-related peptide-binding sites of porcine cardiac muscles and coronary arteries: solubilization and characterization. *J. Neurochem.* **52**:1919–1924 (1989).
39. Stangl, D., W. Born, and J. A. Fischer. Characterization and photoaffinity labeling of a calcitonin gene-related peptide receptor solubilized from human cerebellum. *Biochemistry* **30**:8605–8611 (1991).
40. Smith, S. K., and L. E. Limbird. Solubilization of human platelet α-adrenergic receptors: evidence that agonist occupancy of the receptor stabilizes receptor effector interactions. *Proc. Natl. Acad. Sci. USA* **78**:4026–4030 (1981).
41. Limbird, L. E., D. M. Gill, and R. J. Lefkowitz. Agonist-promoted coupling of the β-adrenergic receptor with the guanine nucleotide regulatory protein of the adenylate cyclase system. *Proc. Natl. Acad. Sci. USA* **77**:775–779 (1980).
42. Kilpatrick, B. F., and M. G. Caron. Agonist binding promotes a guanine nucleotide reversible increase in the apparent size of the bovine anterior pituitary dopamine receptors. *J. Biol. Chem.* **258**:13528–13534 (1983).
43. Niznik, H. B., N. Y. Otsuka, A. Dumbrille-Ross, D. Grigoriadis, A. Tirpak, and P. Seeman. Dopamine D_1 receptors characterized with [^{3}H]SCH 23390. *J. Biol. Chem.* **261**:8397–8406 (1986).
44. Matesic, D. F., D. R. Manning, B. B. Wolfe, and G. R. Luthin. Pharmacological and biochemical characterization of complexes of muscarinic acetylcholine receptor and guanine nucleotide-binding protein. *J. Biol. Chem.* **264**:21638–21645 (1989).
45. Law, S. F., D. Manning, and T. Reisine. Identification of the subunits of GTP-binding proteins coupled to somatostatin receptors. *J. Biol. Chem.* **266**:17885–17897 (1991).
46. Masters, S. B., K. A. Sullivan, R. T. Miller, B. Beiderman, N. G. Lopez, J. Ramachandran, and H. R. Bourne. Carboxyl terminal domain of $G_{s\alpha}$ specifies coupling of receptors to stimulation of adenylyl cyclase. *Science (Washington D. C.)* **241**:448–451 (1988).
47. Robishaw, J. D., M. D. Smigel, and A. G. Gilman. Molecular basis for two forms of the G protein that stimulates adenylate cyclase. *J. Biol. Chem.* **261**:9587–9590 (1986).
48. Stiles, G. L. The A_1 adenosine receptor: solubilization and characterization of a guanine nucleotide-sensitive form of the receptor. *J. Biol. Chem.* **260**:6728–6732 (1985).
49. Jeffs, R. A., C. L. Cooper, and T. K. Harden. Solubilization of a guanine nucleotide-sensitive form of the P_{2Y}-purinergic receptor. *Mol. Pharmacol.* **40**:85–92 (1991).
50. Kermode, J. C., A. W. DeLuca, A. Zilberman, J. Valliere, and S. M. Shreeve. Evidence for the formation of a functional complex between vasoactive intestinal peptide, its receptor, and G_s in lung membranes. *J. Biol. Chem.* **267**:3382–3388 (1992).

Send reprint requests to: Dr. Rory A. Fisher, Department of Pharmacology, The University of Iowa, College of Medicine, Iowa City, IA 52242.