Characterization and Photoaffinity Labeling of a Calcitonin Gene-Related Peptide Receptor Solubilized from Human Cerebellum†

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ABSTRACT: Calcitonin gene-related peptide (CGRP) receptors were solubilized from human (h) cerebellum with use of the zwitterionic detergent 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid (CHAPS). Scatchard analysis of equilibrium binding data indicated that the soluble extract contained a single class of CGRP binding sites with apparent dissociation constants of 50 pM for the intact ^{125}I-hCGRP-I(1–37) and 160 pM for the antagonist ^{125}I-hCGRP-I(8–37). Unlabeled hCGRP-I and -II and hCGRP-I(8–37) displaced ^{125}I-hCGRP-I from solubilized CGRP receptors with similar potencies (ID_{50} = 70–150 pM). Human CGRP-I(15–37), –(21–37), and –(28–37) were less potent ($ID_{50} \geq 70$ nM), suggesting that amino acid residues 8–14 may be important for maintaining high binding affinity. A novel photoreactive analogue of hCGRP-I, ^{125}I-[C$^{\gamma}$-(4-azidoanilino)Asp^3]hCGRP-I, was prepared by carbodiimide coupling of 4-azidoaniline to ^{125}I-hCGRP-I. Photoaffinity labeling of soluble CGRP receptors with the photoreactive analogue and analysis by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and autoradiography revealed three specifically labeled binding proteins with apparent molecular weights (M_r) of 60 000, 54 000, and 17 000. Cross-linking of ^{125}I-hCGRP-I and -II and ^{125}I-hCGRP-I(8–37) to soluble CGRP binding sites using disuccinimidyl suberate revealed three specifically labeled binding proteins with the same M_r. The C-terminal fragment ^{125}I-hCGRP-I(8–37), unlike the intact peptide, was, furthermore, cross-linked specifically to a 95 000 M_r protein. The CGRP receptor is N-glycosylated. Treatment with endoglycosidase F/*N*-glycosidase F converted the 60 000 and 54 000 to 46 000 and 41 000 M_r components. In conclusion, binding and labeling data indicate that the N-terminal ring structure of CGRP is not essential for the interaction with the receptor from the human cerebellum. Moreover, side-chain modification of Asp^3 of hCGRP-I does not interfere with receptor binding.

The calcitonin gene-related peptides (CGRP) I and II (or α and β) are encoded by two separate genes in man and rat (Amara et al., 1985; Jonas et al., 1985; Steenbergh et al., 1986). All known CGRPs have in common a disulfide bridge between cysteines at positions 2 and 7, and the C-terminal phenylalanine is amidated (Morris et al., 1984; Wimalawansa et al., 1990). Human (h) CGRP-I and -II differ in three of the 37 amino acid residues (Figure 1), and they reveal limited structural homology with salmon and human calcitonin (CT) (Fischer & Born, 1985). Human CGRP-I and -II have been identified in the central and peripheral nervous system, pituitary and thyroid glands, and in medullary thyroid carcinoma tissue (Morris et al., 1984; Petermann et al., 1987; Wimalawansa et al., 1990). CGRP is principally a neuropeptide acting locally through interaction with receptors at its target organs. CGRP exerts potent biological actions that include vasodilation (Brain et al., 1985), positive chronotropic and inotropic effects in the heart (Fisher et al., 1983; Franco-Cereceda et al., 1987), and regulation of T-lymphocyte function [Umeda & Arisawa, 1989; for a review, see Born and Fischer (1988)].

The N-terminal fragments hCGRP-I(1–12), -(1–15), and -(1–22) lowered arterial pressure in rats albeit at doses higher than that of the intact peptide (Maggi et al., 1990). The C-terminal fragments, hCGRP-I(8–37) and -(12–37), in contrast, caused vasoconstriction and antagonized vasodilatory effects and the stimulation of the heart by the intact peptide (Dennis et al., 1989, 1990; Han et al., 1990). Similarly, rat [Tyr^0]CGRP(28–37) antagonized the relaxation of the anal smooth musculature and stimulation of amylase release by rat CGRP(1–37) (Chakder & Rattan, 1990; Maton et al., 1990).

Specific CGRP binding sites have been identified in membrane homogenates and by receptor autoradiography in the human and rat central nervous system, heart, and spleen (Tschopp et al., 1985; Goltzman & Mitchell, 1985; Seifert et al., 1985; Skofitsch & Jacobowitz, 1985; Inagaki et al., 1986). Human ^{125}I-hCGRP-I and -II have similar binding characteristics in these tissues. With the use of receptor autoradiography, subtle differences in the regional distribution of ^{125}I-hCGRP-I and -II binding have been found, e.g., in the ventromedial hypothalamus (Henke et al., 1987). Specific ^{125}I-hCGRP-I binding proteins were abundant in the cerebellum and spinal cord. These tissues were therefore an important source for receptor characterization and purification (Dotti-Sigrist et al., 1988; Sano et al., 1989).

Here we describe the solubilization of CGRP receptors from human cerebellum. A novel photoreactive analogue of hCGRP-I ^{125}I-[C$^{\gamma}$-(4-azidoanilino)Asp^3]hCGRP-I, which retained the binding properties of the native peptide, was prepared. With the use of this analogue and the antagonist ^{125}I-hCGRP-I(8–37), four specifically labeled binding proteins have been identified and characterized in soluble extracts from the human cerebellum.

Materials and Methods

Peptides. Synthetic hCGRP-I(1–37) and hCGRP-I(15–37), -(21–37), and -(28–37) were obtained from Bachem (Bubendorf, Switzerland). Human CGRP-I(8–37) was donated by T. Noda (Toyo Jozo Co., Shizuoka Ken, Japan), and

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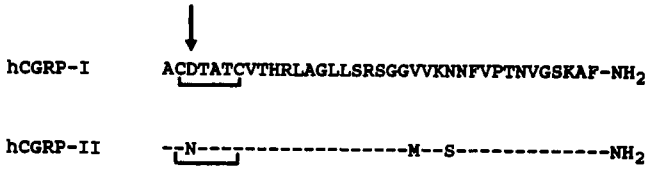

FIGURE 1: Amino acid sequences of hCGRP-I and -II (Morris et al., 1984; Petermann et al., 1987; Wimalawansa et al., 1990). The site of modification with 4-azidoaniline is indicated by the arrow. Horizontal lines represent amino acid residues in common with hCGRP-I.

hCGRP-II(1–37) was purchased from Peninsula Laboratories (Belmont, CA). Human CT was donated by W. Rittel (Ciba-Geigy, Basel, Switzerland) and salmon CT by S. Guttmann (Sandoz, Basel, Switzerland).

The peptides were dissolved in 0.01 M acetic acid and stored in aliquots at −20 °C. The peptide concentration for all peptides used is given as the dry weight of the peptide lot.

Other Materials. 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonic acid (CHAPS), bovine serum albumin (BSA, fraction V), *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine (Tricine), and 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC) were obtained from Sigma (St. Louis, MO); 4-azidoaniline and trifluoroacetic acid (TFA) were from Fluka (Buchs, Switzerland); 2,6-dimethylpyridine-3-sulfonic acid, dithiothreitol (DTT), and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes) were from Merck (Darmstadt, Germany); endoglycosidase F (endo-β-*N*-acetylglucosaminidase F, EC 3.2.1.96) and *N*-glycosidase F (peptide N^4-(*N*-acetyl-β-glucosaminyl)asparagine amidase, EC 3.2.2.18) (Endo F/PNGase F) were from Boehringer (Mannheim, Germany); disuccinimidyl suberate (DSS) was from Pierce (Rockford, IL); dextran T70 and premixed M_r standards for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) were from Pharmacia (Uppsala, Sweden); and sodium [^{125}I]iodide (≈2000 Ci/mmol) and Hyperfilm-βmax were from Amersham (Buckinghamshire, U.K.). All other reagents used were of the highest grade available.

Radioiodination. Human CGRP-I, hCGRP-I(8–37), and hCGRP-II were iodinated to specific activities of approximately 200 Ci/mmol with use of a modified chloramine-T method (Hunter & Greenwood, 1962). Briefly, iodination was performed at room temperature in 30 μL of 0.4 M sodium phosphate, pH 7.3, with a molar ratio for peptide/^{125}I$^-$/chloramine-T of 3:1:200. After 30 s, the reaction was stopped by the addition of a 12-fold molar excess of sodium metabisulfite over chloramine-T followed by 0.5 mL of 10% (v/v) human serum in 0.2 M ammonium acetate, pH 4.7. The reaction mixture was desalted on a Bio-Gel P-30 (Bio-Rad, Richmond, CA) column (1 × 40 cm) equilibrated in 0.2 M ammonium acetate, pH 4.7, containing 0.25% (w/v) BSA, and stored at −20 °C.

Radioligand Purification. ^{125}I-hCGRP-I and -II were purified by HPLC with use of a 4 × 250 mm Nucleosil C_{18} column (10-μm particles, 100-Å pore size; Macherey-Nagel, Dueren, Germany). Aliquots of ^{125}I-hCGRP-I and -II were applied to the column equilibrated in 29% acetonitrile in 0.1% TFA and eluted with a linear gradient of 29–35% acetonitrile in 0.1% TFA over 60 min. The flow rate was 1 mL/min. Purification of ^{125}I-hCGRP-I(8–37) was carried out on the same column equilibrated in 24% acetonitrile in 0.1% TFA and eluted with a linear gradient of 24–32% acetonitrile in 0.1% TFA over 80 min. One-milliliter fractions were collected in glass tubes containing 50 μL of 20 mM CHAPS and 50 mM Hepes, pH 8.

^{125}I-hCGRP-I, ^{125}I-hCGRP-I(8–37), and ^{125}I-hCGRP-II each resolved into a major and a minor peak of radioactivity, all eluting with retention times longer than those of the unlabeled peptides (not shown). The earlier eluting major peak of ^{125}I-hCGRP-I radioiodinated in our laboratory had the retention time of monoiodinated [2-[^{125}I]iodohistidyl^{10}]-hCGRP-I purchased from Amersham (Buckinghamshire, U.K.), and the binding characteristics of the two peptides were indistinguishable. The later eluting radioactive peak probably corresponds to diiodinated hCGRP-I. Specific binding of the later eluting peaks of ^{125}I-hCGRP-I, -II, and -I(8–37) to solubilized CGRP receptors was twice as high compared to those of the corresponding earlier eluting peaks. The later eluting peaks were therefore used for chemical cross-linking experiments and the preparation of the photoreactive analogue. The earlier eluting peaks of ^{125}I-hCGRP-I and ^{125}I-hCGRP-I(8–37) were used for saturation binding experiments.

Carbodiimide Coupling of 4-Azidoaniline to ^{125}I-hCGRP-I. All procedures were performed under safe light conditions. A total of 1–2 pmol of HPLC purified, lyophilized ^{125}I-hCGRP-I was dissolved in 0.9 mL of coupling buffer (0.5 M 2,6-dimethylpyridine-3-sulfonic acid, pH 4.7). 4-Azidoaniline was added at a final concentration of 30 mM. The coupling reaction was carried out with 0.1 mL of a fresh solution of 1 M EDC in coupling buffer at room temperature for 3 h in the dark with occasional vortexing. The reaction mixture was then applied to a Sep-Pak C_{18} cartridge (Waters, Milford, MA) equilibrated in acetonitrile/water/TFA 20:79.9:0.1 (v/v/v). The cartridge was washed with 15 mL of the same buffer to remove excess reagents and then eluted with acetonitrile/water/formic acid 80:19:1 (v/v/v) into a siliconized glass test tube containing 50 μL of 20 mM CHAPS and 50 mM Hepes, pH 8. The eluate containing the photoreactive analogue of hCGRP-I was lyophilized and stored at −20 °C in the dark. Preparation of the unlabeled, photoreactive analogue was carried out with use of the same procedure.

Solubilization of CGRP Binding Proteins. Human cerebellum was obtained at autopsy within 12 h post mortem and stored frozen at −80 °C until used. Homogenization was carried out as described (Dotti-Sigrist et al., 1988). Briefly, minced human cerebellum was homogenized with use of an Elvejehm potter in 3 volumes of ice-cold 0.25 M sucrose/1 mM EDTA/5 mM Tris, pH 7.4. The homogenate was centrifuged at 1500*g* for 10 min. The pellet was resuspended in 2 M sucrose/1 mM EDTA/5 mM Tris, pH 7.4, rehomogenized, and centrifuged at 13000*g* for 10 min. The supernatant without the lipid layer on top was diluted 8-fold in 1 mM EDTA/5 mM Tris, pH 7.4, and centrifuged at 20000*g* for 15 min. The final pellet was resuspended in 20% (w/v) glycerol/50 mM Hepes, pH 8, at 10–15 mg of protein/mL. The binding proteins were solubilized with 5 mM CHAPS at 4 °C for 2 h on a slowly rotating device. The suspension was then centrifuged at 100000*g* for 60 min, and the supernatant was used as the source of soluble CGRP binding proteins.

Binding Studies. Binding and displacement studies were performed with 15–25 μg of solubilized proteins and 40–50 fmol of radiolabeled hCGRP-I not purified by HPLC. For saturation binding experiments, HPLC purified ^{125}I-hCGRP-I and ^{125}I-hCGRP-I(8–37) (≈2000 Ci/mmol) were used. Binding assays were performed for 2 h in polystyrene tubes at 4 °C in 0.4 mL of 20% (w/v) glycerol/5 mM CHAPS/1 mM EDTA/50 mM Hepes, pH 8. Incubation was terminated by the addition of 2 mL of an ice-cold suspension of dextran-coated charcoal [0.25% (w/v) dextran T70, 2.5% (w/v) Norit (Fisher Scientific Co., NJ), 1% (w/v) BSA, 0.13 M

NaCl, 7 mM sodium acetate, and 7 mM sodium barbital, pH 7.4). The samples were then centrifuged at 1500g at 4 °C for 10 min. The supernatants containing receptor-bound radiolabeled CGRP were counted in a MR 252 γ-counter (Kontron, Zurich, Switzerland).

Specific binding is defined as the difference between total binding and binding in the presence of 0.6 μM unlabeled peptide. Under the incubation conditions used, total binding was approximately 30% and nonspecific binding was less than 5%.

Photoaffinity Labeling of CGRP Receptors. Aliquots of solubilized CGRP receptors (≈60 μg of total protein) were added to 0.5 mL of 20% (w/v) glycerol/5 mM CHAPS/1 mM EDTA/20 mM Hepes, pH 8, in siliconized glass test tubes and incubated with 20–30 fmol of ^{125}I-labeled [C$^{\gamma}$-(4-azidoanilino)Asp^3]hCGRP-I in the absence and presence of 0.5 μM unlabeled peptides in the dark on ice for 2 h. DTT was then added to a final concentration of 1 mM to reduce nonspecific labeling. After 5 min, the samples were irradiated for 15 min on ice with a Spectroline SB-100 long-wave UV lamp (emission maximum 356 nm; Spectronics Corp., Westbury, NY) at a source to sample distance of 5–7 cm.

Affinity Labeling of CGRP Receptors by Chemical Cross-Linking with DSS. A total of 20–40 fmol of HPLC purified ^{125}I-hCGRP-I, ^{125}I-hCGRP-II, or ^{125}I-hCGRP-I(8–37) was added to aliquots of solubilized CGRP receptors (≈60 μg of total protein) in 0.5 mL of 20% (w/v) glycerol/5 mM CHAPS/1 mM EDTA/20 mM Hepes, pH 8. The samples were then incubated for 90 min on ice in the absence and presence of 0.5 μM unlabeled peptides (salmon CT, hCGRP-I, hCGRP-II, hCGRP-I(8–37)). Cross-linking of the ligand–receptor complexes was initiated by the addition of 5 μL of 50 mM DSS in dimethyl sulfoxide. After 15 min on ice, the reaction was terminated with 15 μL of 2.5 M Tris, pH 6.8.

Endo F/PNGase F Treatment of CGRP Receptors. Samples of photoaffinity labeled or DSS-cross-linked CGRP binding proteins in 0.6 mL of 20% (w/v) glycerol/5 mM CHAPS/5 mM EDTA/1% (w/v) Triton X-100/0.1% (w/v) SDS/20 mM Hepes/50 mM 2,6-dimethylpyridine-3-sulfonic acid, pH 5, were mixed with 0.2 unit of Endo F/PNGase F and incubated at 37 °C for 17 h. Control samples were incubated without Endo F/PNGase F.

SDS–PAGE. After photoaffinity labeling or chemical cross-linking of solubilized CGRP binding proteins with DSS, samples were precipitated with a methanol/chloroform/water mixture according to the method of Wessel and Flügge (1984), lyophilized, and dissolved in electrophoresis sample buffer [4% (w/v) SDS, 12% (w/v) glycerol, 2% (v/v) β-mercaptoethanol, 0.01% (w/v) Serva Blue G, and 50 mM Tris, pH 6.8]. Electrophoresis was carried out with the use of the discontinuous Tricine system described by Schägger and von Jagow (1987). Separating gels contained 10% (w/v) acrylamide and 0.3% (w/v) bisacrylamide. M_r standards used were phosphorylase b (94 000), albumin (67 000), ovalbumin (43 000), carbonic anhydrase (30 000), trypsin inhibitor (20 100), and α-lactalbumin (14 400). After electrophoresis, the gels were stained with Serva Blue R, destained, and vacuum-dried. Dried gels were exposed to Hyperfilm-βmax and intensifying screens for 5–11 days at −80 °C.

Sequence Analysis. Sequencing of hCGRP-I and its photoreactive analogue [C$^{\gamma}$-(4-azidoanilino)Asp^3]hCGRP-I was performed with use of a Model 470A sequencer (Applied Biosystems, Foster City, CA). Samples were loaded on precycled polybrene-coated glass fiber filters in the dark and protected from light throughout the procedure. Phenylthiohydantoin- (PTH-) amino acids were identified by isocratic reversed-phase HPLC on a Spherisorb ODS2 5-μm column (4 × 250 mm) at 37 °C as described by Frank (1989).

Results

Preparation of the Photoreactive Analogue of hCGRP-I ^{125}I-[C$^{\gamma}$-(4-Azidoanilino)Asp^3]hCGRP-I. 4-Azidoaniline was coupled to Asp^3 of HPLC purified ^{125}I-hCGRP-I by the use of the water-soluble carbodiimide EDC at pH 4.7. HPLC analysis of the reaction mixture after incubation for 3 h revealed no radioactivity at the position of the nonmodified peptide, but a predominant peak of radioactivity eluted with a longer retention time (not shown).

Automated Edman degradation of the unlabeled photoreactive analogue revealed no measurable PTH-Asp in the third degradation cycle. Instead, a component eluting near PTH-Phe was recognized. Undesired condensation reactions of the carbodiimide coupling procedure may include inter- and intramolecular cross-linking of the β-carboxyl group of Asp^3 with the N-terminal amino group and the ϵ-amino groups of Lys^{24} and Lys^{35}. Intermolecular cross-linking probably does not occur in view of the large excess of reactive (unprotonated) amino groups of 4-azidoaniline over the peptide amino groups at pH 4.7. Intramolecular cross-linking may occur due to locally high concentrations of peptide amino groups, yet sequence analysis gave no indication of a blocked N-terminus or a modified lysine residue at cycle 24. Identification of Lys^{35} was not possible because of an insufficient yield of PTH-amino acids. Apparently, 4-azidoaniline was coupled with high yield to the β-carboxyl group of Asp^3.

Solubilization of CGRP Receptors. Membrane homogenates from human cerebellum were solubilized with 5 mM zwitterionic detergent CHAPS. Approximately 20% of the total membrane proteins and ^{125}I-hCGRP-I binding activity were recovered. Binding activity of solubilized CGRP binding proteins was maintained at 4 °C in the presence of 20% (w/v) glycerol for at least 6 days. In the absence of glycerol, binding activity was lowered to 50% within 6 days.

Ligand Binding Characteristics. Specific binding of ^{125}I-hCGRP-I to solubilized CGRP receptors was maximal at pH 7–9 at 4 °C. Specific binding was not detected below pH 5 or above pH 10. The addition of DTT (0.5 mM) or sodium chloride (100 mM) lowered specific binding by over 50%. Saturable binding was obtained with HPLC purified ^{125}I-hCGRP-I and ^{125}I-hCGRP-I(8–37). Scatchard analysis revealed a single class of binding sites with maximal concentrations (B_{max}) and dissociation constants (K_d) of 1 pmol/mg of protein and 50 pM for ^{125}I-hCGRP-I and 0.3 pmol/mg of protein and 160 pM for ^{125}-hCGRP-I(8–37) (not shown).

Half-maximal inhibition of specific ^{125}I-hCGRP-I binding was obtained with 150 pM hCGRP-I, 300 pM [C$^{\gamma}$-(4-azidoanilino)Asp^3]hCGRP-I, 150 pM hCGRP-II, 70 pM hCGRP-I(8–37), 70 nM hCGRP-I(15–37), 300 nM hCGRP-I(21–37), 1.5 μM salmon CT, and 10 μM hCGRP-I(28–37) (Figure 2). Inhibition of binding by the photoreactive analogue of hCGRP-I [C$^{\gamma}$-(4-azidoanilino)Asp^3]-hCGRP-I and by hCGRP-I(8–37) was similar to that of hCGRP-I and -II. The shorter C-terminal fragments of hCGRP-I displaced radiolabeled hCGRP-I with decreasing potencies in parallel to their size. Inhibition of binding was obtained with a 10 000-fold higher concentration of salmon CT than hCGRP-I, and human CT did not inhibit binding at up to 50 μM.

Photoaffinity Labeling of CGRP Receptors. Solubilized CGRP binding proteins were photochemically labeled with ^{125}I-[C$^{\gamma}$-(4-azidoanilino)Asp^3]hCGRP-I. SDS-PAGE and

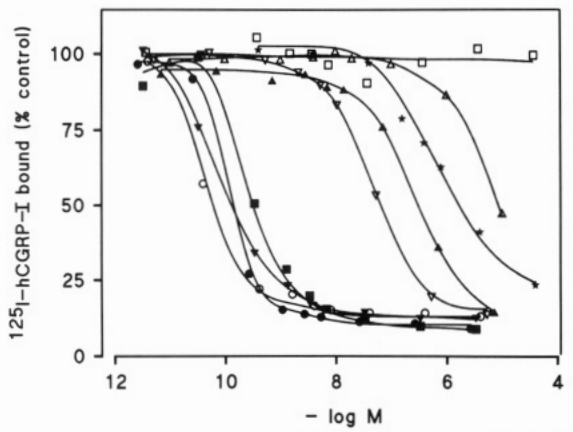

FIGURE 2: Displacement of ^{125}I-hCGRP-I binding to solubilized CGRP receptors by hCGRP-I (●), [C$^{\gamma}$-(4-azidoanilino)Asp^3]hCGRP-I (■), hCGRP-II (▼), hCGRP-I(8–37) (○), hCGRP-I(15–37) (▽), hCGRP-I(21–37) (▲), hCGRP-I(28–37) (△), salmon CT (★), and human CT (□). Incubations were carried out at 4 °C for 2 h. Each point represents the mean of triplicate determinations of a representative experiment.

autoradiography revealed three specifically labeled binding components with apparent molecular weights of 60 000, 54 000, and 17 000 (Figure 3). The 17 000 M_r component was visible after prolonged exposure of the autoradiograms. Photoaffinity labeling was reduced in the presence of unlabeled hCGRP-I in a concentration-dependent manner. In the presence of an excess (0.5 μM) of unlabeled hCGRP-I and -II and the photoreactive analogue of hCGRP-I, labeling of the three binding proteins was decreased or abolished. In contrast, labeling was not affected by 0.5 μM salmon CT. The patterns of the labeled binding proteins were identical under reducing (50 mM DTT) and nonreducing conditions (Figure 4).

Photoaffinity labeled CGRP binding proteins were treated with Endo F/PNGase F, a mixture of two endoglycosidases releasing N-linked oligosaccharides from the peptide backbone (Maley et al., 1989). After incubation at 37 °C for 17 h, the labeled CGRP binding proteins with M_r values of 60 000 and 54 000 were converted to components with M_r values of 46 000 and 41 000 (Figure 4).

No high molecular weight binding proteins were detected on 5–15% gradient gels [according to Laemmli (1970)] under reducing as well as nonreducing conditions. With this gradient gel system the apparent molecular weights of the 60 000 and 54 000 components determined with the Tricine gel system were reduced to 54 000 and 47 000, respectively (not shown).

Cross-Linking of CGRP Receptors to ^{125}I-hCGRP-I and -II and ^{125}I-hCGRP-I(8–37) with DSS. The patterns of the specifically labeled 60 000, 54 000, and 17 000 CGRP binding proteins cross-linked to ^{125}I-hCGRP-I or -II were indistinguishable (Figure 5). Treatment with Endo F/PNGase F of the binding proteins cross-linked to ^{125}I-hCGRP-I and -II converted the 60 000 and 54 000 to 46 000 and 41 000 M_r components. The C-terminal fragment ^{125}I-hCGRP-I(8–37) was similarily cross-linked to soluble CGRP receptors, but an additional 95 000 M_r binding protein was specifically labeled. An excess of hCGRP-I(8–37) and of intact hCGRP-I abolished labeling of this binding protein. Treatment with Endo F/PNGase F did not alter the size of the 95 000 M_r component.

Discussion

CGRP receptors from human cerebellum have been solubilized in an active and stable form with use of the zwitterionic detergent CHAPS. Receptor binding was not affected by the solubilization procedure used. Calculated K_d values for ^{125}I-hCGRP-I binding to solubilized and particulate CGRP receptors from human cerebellum were similar (Henke et al., 1987). The specificity of the binding to solubilized and particulate receptors was the same with ^{125}I-hCGRP-I and -II.

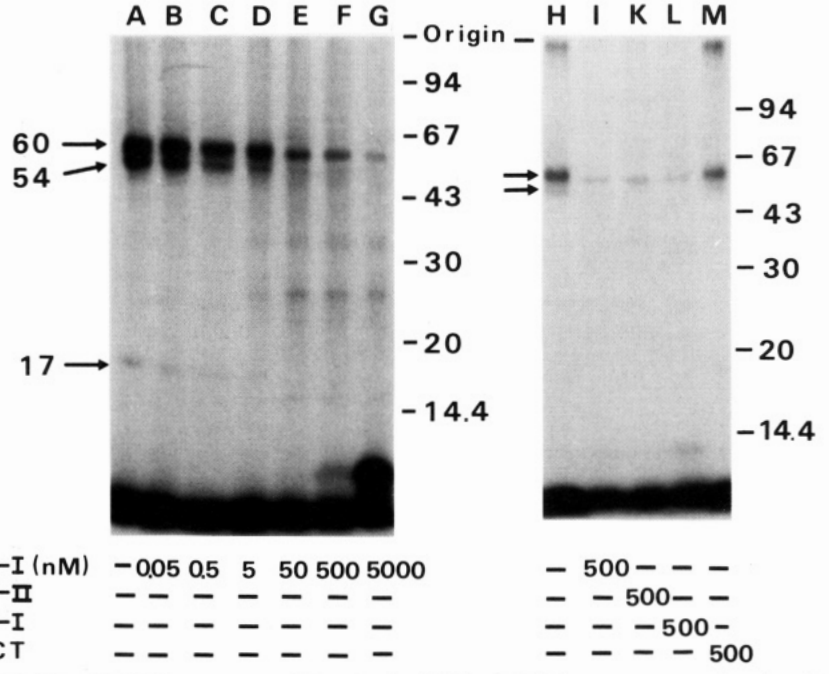

FIGURE 3: Photochemical affinity labeling of CGRP receptors. Aliquots of solubilized CGRP receptors were incubated on ice for 2 h in the dark with ^{125}I-[C$^{\gamma}$-(4-azidoanilino)Asp^3]hCGRP-I in the absence (lanes A and H) and presence of increasing concentrations (nanomolar) of hCGRP-I (lanes B–G), 500 nM hCGRP-I (lane I), 500 nM hCGRP-II (lane K), 500 nM *hCGRP-I ([C$^{\gamma}$-(4-azidoanilino)Asp^3]hCGRP-I, lane L), and 500 nM salmon CT (lane M). DTT was added to a final concentration of 1 mM 5 min prior to photolysis. The samples were then photolyzed followed by precipitation with a methanol/chloroform/water mixture as described under Materials and Methods. SDS-denatured samples were analyzed by SDS–PAGE (Tricine system, 10%) under reducing conditions, and radiolabeled components were identified after autoradiography at −80 °C for 5 days. Arrows point to protein bands of interest (apparent $M_r \times 10^{-3}$). The M_r standards ($\times 10^{-3}$) are indicated. Without photolysis no protein bands were detected (not illustrated).

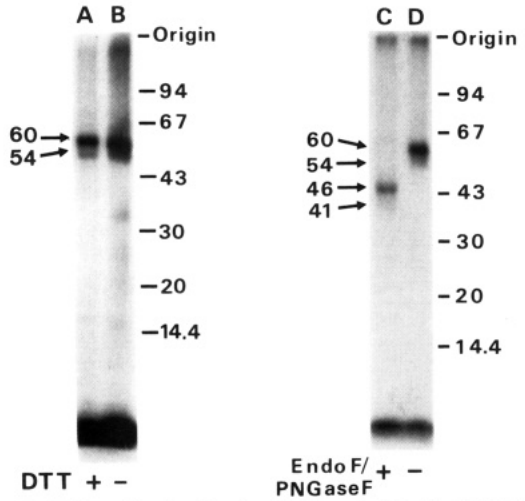

FIGURE 4: Effect of reduction by DTT and of Endo F/PNGase F treatment on photoaffinity labeled CGRP receptors. Solubilized CGRP binding proteins were photoaffinity labeled in the presence (lane A) and absence of 1 mM DTT (lane B). SDS–PAGE (Tricine system, 10%) was carried out under reducing (50 mM DTT, lane A) and nonreducing (lane B) conditions. Photoaffinity labeled CGRP binding proteins were incubated at pH 5 for 17 h at 37 °C in the presence (lane C) and absence (lane D) of Endo F/PNGase F (0.4 unit/mL). Subsequently, the samples were precipitated with a mixture of methanol/chloroform/water and subjected to SDS–PAGE under reducing conditions as described under Materials and Methods. Autoradiography was performed at −80 °C for 6 days. The M_r standards ($\times 10^{-3}$) are indicated.

CHAPS solubilized CGRP receptors were characterized with respect to binding of the C-terminal fragment hCGRP-I(8–37) and of intact hCGRP-I. Scatchard analysis of ^{125}I-hCGRP-I(8–37) binding revealed a lower B_{max} and somewhat higher K_d compared to ^{125}I-hCGRP-I(1–37). But hCGRP-I(8–37) displaced ^{125}I-hCGRP-I from the receptors with the potency of intact hCGRP-I. These results are similar to those of Dennis et al. (1990), who showed that hCGRP-I(8–37) was slightly more potent than hCGRP-I in displacing ^{125}I-hCGRP-I from membrane preparations of the rat central nervous system and spleen and the heart atrium and vas deferens of the guinea pig. The 1000-fold higher ID_{50} of hCGRP-I(8–37) compared to that of hCGRP-I(1–37) in the rat liver is evidence of a different CGRP receptor or may be caused by the rat ^{125}I-[Tyr^0]CGRP used as radioligand (Chiba et al., 1989). The smaller C-terminal fragments hCGRP-I-(15–37), -(21–37), and -(28–37) were over 1000 times less potent than hCGRP-I(8–37) in displacing ^{125}I-hCGRP-I from solubilized proteins of the human cerebellum. The results suggest that amino acid residues between positions 8 and 14 contribute to the high binding affinity of hCGRP-I and hCGRP-I(8–37). They form part of an amphipathic α-helix between positions 8 and 18 proposed by Lynch and Kaiser (1988) for rat CGRP-I, and expected for hCGRP-I as well. Indeed, measurements of circular dichroism of hCGRP-I in aqueous solution at 4 °C revealed some α-helical structure involving 8–10 amino acid residues (Manning, 1989).

Modification of Asp^3 of ^{125}I-hCGRP-I with 4-azidoaniline shown here or *N*-biotinyl-1,8-diamino-3,6-dioxaoctane (unpublished observation) and the naturally occurring substitution of Asp^3 by Asn in hCGRP-II were without consequences for binding to the solubilized CGRP receptors. These findings indicate that side-chain modifications of Asp^3 located within the N-terminal ring structure of hCGRP-I are well tolerated with respect to receptor binding. Analogues of hCGRP-I modified at Asp^3 with, e.g., photoreactive, fluorescent, or biotin-containing groups are potentially important tools for CGRP receptor characterization and isolation. To this end, hCGRP-I immobilized via carbodiimide coupling of Asp^3 to a suitable solid support may be used for the purification of CGRP receptors by ligand affinity chromatography.

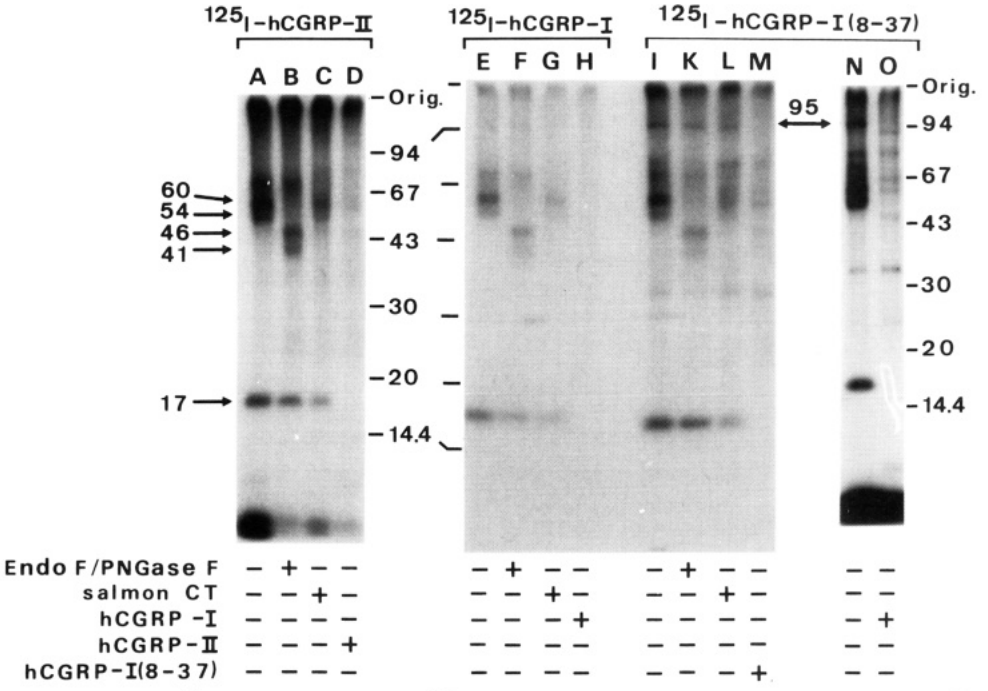

FIGURE 5: Chemical cross-linking of ^{125}I-hCGRP-I and -II and ^{125}I-hCGRP-I(8–37) to solubilized CGRP receptors using DSS. Solubilized CGRP receptors were incubated for 2 h on ice with HPLC purified ^{125}I-hCGRP-II (lanes A–D), ^{125}I-hCGRP-I (lanes E–H), and ^{125}I-hCGRP-I(8–37) (lanes I–O) in the absence and presence of 0.5 μM hCGRP-II (lane D), hCGRP-I (lanes H and O), hCGRP-I(8–37) (lane M), and salmon CT (lanes C, G, and L). After cross-linking with 0.5 mM DSS, samples were precipitated with a mixture of methanol/chloroform/water and subjected to SDS–PAGE (Tricine system, 10%) under reducing conditions as described under Materials and Methods. Prior to precipitation, samples of lanes B, F, and K were treated with Endo F/PNGase F (0.4 unit/mL) at pH 5 for 17 h at 37 °C. Autoradiography was performed for 10 days at −80 °C. Specifically labeled ligand–protein components are indicated by arrows. M_r standards ($\times 10^{-3}$) are indicated.

Covalent labeling of solubilized CGRP receptors from human cerebellum with photoreactive ^{125}I-[C$^{\gamma}$-(4-azidoanilino)Asp^3]hCGRP-I and with ^{125}I-hCGRP-I and -II revealed binding proteins with apparent M_r values of 60 000, 54 000, and 17 000 after SDS–PAGE using the Tricine system. On gradient gels according to Laemmli (1970), the same photoaffinity labeled binding proteins had apparent M_r values of 54 000, 47 000, and 17 000. The 54 000 and 17 000 components were indistinguishable from the earlier reported binding proteins using the same gel system (Dotti-Sigrist et al., 1988). The 47 000 M_r protein corresponded to the 54 000 M_r protein identified with the presently used Tricine gel system. It was not detected when particulate CGRP receptors were cross-linked to ^{125}I-hCGRP-I and -II with DSS (Dotti-Sigrist et al., 1988).

Recently, a CGRP binding protein with a M_r of 60 000 was recognized in cultured rat vascular smooth muscle cells and in bovine endothelial cells by cross-linking with DSS (Hirata et al., 1988). Specific CGRP binding proteins were also identified in porcine coronary arteries (M_r 90 000 and 70 000) and in the heart atrium and ventricular muscles (M_r 120 000 and 70 000) (Miyauchi et al., 1988; Sano et al., 1989). In the porcine spinal cord the 70 000 M_r CGRP binding protein was identified alone (Sano et al., 1989). The M_r values of the CGRP binding proteins described so far were the same under reducing and nonreducing conditions.

Treatment of labeled CGRP binding proteins from the human cerebellum with Endo F/PNGase F demonstrated that the 60 000 and 54 000 M_r components are N-glycosylated. Their apparent molecular weights were reduced by one-fourth. In contrast, the M_r 95 000 and 17 000 components were not affected by Endo F/PNGase F treatment. The 95 000 M_r binding protein that was recognized with ^{125}I-hCGRP-I(8–37) only is probably a distinct binding component and not the result of intermolecular cross-linking involving a glycosylated binding protein. Isolation and gene cloning are required for a more detailed structural analysis of the CGRP receptor.

In conclusion, a CGRP receptor has been solubilized from human cerebellum in an active and stable form. With a novel photoreactive analogue of hCGRP-I derivatized at Asp^3, two N-glycosylated CGRP binding proteins with apparent M_r values of 60 000 and 54 000 have been identified. The same binding proteins have been recognized through chemical cross-linking of ^{125}I-hCGRP-I(1–37), -II(1–37), and -I(8–37). The N-terminal ring structure is not crucial for binding to the receptor proteins. Agonist binding of the intact hCGRP-I-(1–37) and antagonist binding of hCGRP-I(8–37) are indistinguishable.

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Registry No. CGRP, 83652-28-2; hCGRP-I, 90954-53-3; hCGRP-II, 101462-82-2; hCGRP-I (8–37), 119911-68-1; hCGRP-I (15–37), 134905-20-7; hCGRP-I (21–37), 134905-21-8; hCGRP-I (28–37), 134905-22-9; salmon CT, 47931-85-1; [C$^{\gamma}$-(4-azidoanilino)Asp^3]-hCGRP-I, 135041-36-0; 4-azidoaniline, 14860-64-1.

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Identification of the V_1 Vasopressin Receptor by Chemical Cross-Linking and Ligand Affinity Blotting†

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ABSTRACT: Chemical and photoaffinity cross-linking experiments as well as ligand affinity blotting techniques were used to label the V_1 vasopressin receptor. In order to determine the optimal reaction conditions, pig liver membranes were incubated with 5 nM [8-lysine]vasopressin (LVP) labeled with ^{125}I and then cross-linked with the use of DMS (dimethyl suberimidate), EGS [ethylene glycol bis(succinimidyl succinate)] or HSAB (hydroxysuccinimidyl *p*-azidobenzoate) at different final concentrations. Consistently, EGS was found to label with high yield one band of M_r 60000 in rat and pig liver membranes when used at a final concentration between 0.05 and 0.25 mM. The protein of M_r 60000 is labeled in a concentration-dependent manner when pig liver membranes are incubated with increasing concentrations of ^{125}I-LVP and then cross-linked with EGS. The label was displaced by increasing concentrations of unlabeled LVP or d(CH_2)$_5$[Tyr^2(Me),-Tyr^9(NH_2)]AVP (V_1/V_2 antagonist). A protein band of similar molecular mass was cross-linked with ^{125}I-LVP in rat liver membranes. The reaction was specific since the incorporation of label into the protein of M_r 60000 was inhibited by LVP, [8-arginine]vasopressin (AVP), the V_1/V_2-antagonist, and the specific V_1-antagonist d(CH_2)$_5$[Tyr^2(Me)]AVP, only partially by [des-Gly^9]AVP (V_2-agonist) and by oxytocin, and not at all by angiotensin II. Incubation of nitrocellulose containing membrane proteins from pig liver with ^{125}I-LVP showed the labeling of a band of M_r 58000 that is inhibited by an excess of unlabeled LVP. This band of M_r 58000 seems to correspond with the protein of M_r 60000 revealed by the cross-linking experiment. This protein appears not to have internal disulfide bonds since the electrophoretic pattern did not change in the absence or presence of reductant in the polyacrylamide gel electrophoresis. Our results suggest that the V_1 vasopressin receptor is a monomeric protein of M_r 60000.

Vasopressin, which is secreted into the systemic circulation in the neural lobe (Pickering et al., 1986), exhibits a wide range of biological activities. In addition to antidiuresis and vasoconstriction, these activities include regulation of glucose metabolism (Hems & Whitton, 1973; Cantau et al., 1980), platelet aggregation (Haslam & Rosson, 1972), regulation of the motility of epididymis and vas deferens (Jaakkola & Talo, 1981), and modulation of neuronal function (De Wied et al., 1978). These diverse biological effects are produced by the interaction of the hormone with at least two types of receptors (Michell et al., 1979). The V_1 type receptor stimulates the formation of 1,2-diacylglycerol and inositol 1,3,4-triphosphate, thus increasing cytosolic Ca^{2+}, whereas the V_2 type receptor activates the formation of cyclic AMP (De Wulf et al., 1980; Jard, 1983a; Guillon et al., 1986). The V_1 type receptor has been identified in the liver (Cantau et al., 1980), in the vascular smooth muscle (Penit et al., 1983), in platelets (Siess et al., 1986), in some portions of the male genital tract (Maggi et al., 1987), and in the brain (Pearlmutter et al., 1983). The V_2 type receptor has been found in the renal medulla (Bockaert et al., 1973) and in the seminal vesicles (Maggi et al., 1988).

An understanding of the events in vasopressin receptor activation and function involves the identification and molecular characterization of the different components of the receptor system.

Conflicting evidence has been reported concerning the molecular structure of the vasopressin receptor. Apparent

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