Functional Calcitonin Gene-Related Peptide Subtype 2 Receptors in Porcine Coronary Arteries Are Identified as Calcitonin Gene-Related Peptide Subtype 1 Receptors by Radioligand Binding and Reverse Transcription-Polymerase Chain Reaction

BOYD R. RORABAUGH, MARGARET A. SCOFIELD, D. DAVID SMITH, WILLIAM B. JEFFRIES, and PETER W. ABEL

Department of Pharmacology (B.R.R., M.A.S., W.B.J., P.W.A.) and Department of Biomedical Sciences (D.D.S.), Creighton University School of Medicine, Omaha, Nebraska

Received June 29, 2001; accepted August 28, 2001

This paper is available online at http://jpet.aspetjournals.org

ABSTRACT

Calcitonin gene-related peptide (CGRP) receptors are classified into CGRP subtype 1 (CGRP1) and CGRP subtype 2 (CGRP2) based on the affinity of the antagonist, human α (hu)-CGRP8–37. hu-CGRP8–37 antagonizes CGRP1 receptor-mediated responses with high affinity (Kd < 100 nM) and antagonizes CGRP2 receptor-mediated responses with low affinity (Kd > 1 μM). CGRP2 receptors have been previously reported to mediate relaxation of large porcine coronary arteries because this action is antagonized with low affinity by hu-CGRP8–37. In the present study, we used reverse transcription-polymerase chain reaction, radioligand binding, and values from our previously reported isolated tissue experiments to compare the CGRP receptor in porcine coronary arteries with the porcine CGRP1 receptor stably expressed in human embryonic kidney (HEK) 293 cells. We identified calcitonin receptor-like receptor and receptor activity modifying protein 1 mRNA in coronary arteries. We also found that the ligand binding characteristics of the CGRP receptor in coronary arteries and the cloned CGRP1 receptor were highly similar. Kd values for hu-CGRP8–37 were 6.6 and 5.7 nM in porcine coronary arteries and the cloned CGRP1 receptor, respectively. The affinities (Kd) of hu-CGRP8–37 and five other antagonists were 22- to 707-fold lower in functional experiments measuring relaxation of coronary arteries than in radioligand binding experiments. Despite this difference in absolute affinity values, there was a high correlation of the rank order of affinity for the antagonists determined by the two methods. Thus hu-CGRP8–37 antagonizes CGRP-induced relaxation of porcine coronary arteries with low affinity at the CGRP1 receptor. Taken together, these data do not support the existence of the CGRP2 receptor.

α-Calcitonin gene-related peptide (CGRP) is a 37-amino acid peptide generated by alternative splicing of the calcitonin gene primary transcript (Amara et al., 1982). β-CGRP, a second form of this peptide, is the product of a separate gene and varies from α-CGRP by three amino acids (Steenbergh et al., 1985). CGRP is widely distributed in central and peripheral regions of the nervous system and is involved in nociception (Yu et al., 1998), appetite suppression (Tannenbaum and Gotzmann, 1985), and regulation of gastrointestinal motility (Raybould, 1992). CGRP has also been proposed to play a role in inflammatory responses (Smith et al., 1993; Kilo et al., 1997), wound healing (Engin, 1998), and the maintenance of vascular tone (Gangula et al., 2000). Therapeutic uses for CGRP and its analogs are currently under investigation for the treatment of migraine headache (Doods et al., 2000) and other disorders.

CGRP produces its effects by activating specific G-protein-coupled receptors at the cell surface. Based on isolated tissue

ABBREVIATIONS: CGRP, calcitonin gene-related peptide; Cha, cyclohexylalanine; CRLR, calcitonin receptor-like receptor; CGRP1, calcitonin gene-related peptide subtype 1; CGRP2, calcitonin gene-related peptide subtype 2; hu-CGRP, human α-calcitonin gene-related peptide; HEK, human embryonic kidney; [125I]hu-CGRP, 2-iodohistidyl10hu-CGRP; IC50, ligand concentration that inhibits 50% of the radioligand bound at equilibrium; Kd, functional equilibrium dissociation constant; Kd, kinetic equilibrium dissociation constant; Kassoc, association rate constant; Kobs, observed association rate constant; Kdissoc, dissociation rate constant; PCR, polymerase chain reaction; RAMP 1, receptor activity modifying protein 1; RT-PCR, reverse transcription-polymerase chain reaction.
studies with the antagonist ho-CGRP<sub>8–37</sub>, two CGRP receptor subtypes, CGRP<sub>1</sub> and the putative CGRP<sub>2</sub> receptor, have been proposed to mediate the effects of CGRP. ho-CGRP<sub>8–37</sub> reportedly antagonizes CGRP<sub>1</sub> receptor-mediated responses with high affinity (K<sub>B</sub> < 100 nM) and antagonizes CGRP<sub>2</sub> receptor-mediated responses with low affinity (K<sub>B</sub> > 1 μM) (Dennis et al., 1990; Mimeault et al., 1991; Wissskirchen et al., 1998). Agonists have also been used to classify CGRP receptors. It has been reported that [Cys(ACM)<sup>2,7</sup>]ho-CGRP is an agonist at the CGRP<sub>2</sub> receptor and is inactive at the CGRP<sub>1</sub> receptor (Dennis et al., 1989; Wissskirchen et al., 1998). These data are consistent with the existence of two CGRP receptor subtypes.

The CGRP<sub>1</sub> receptor is formed by the coexpression of the calcitonin receptor-like receptor (CRLR) and receptor activity modifying protein 1 (RAMP 1) (McLatchie et al., 1998). In nearly all tissues and cells that contain CGRP<sub>1</sub> receptors, this receptor is coupled to an increase in intracellular 3',5' cyclic adenosine monophosphate. Activation of the CGRP<sub>1</sub> receptor is also reported to cause intracellular calcium mobilization (Aiyar et al., 1999) and the activation of extracellular regulated kinase, P-38 mitogen-activated protein kinase, and Jun kinase in some systems (Disa et al., 2000; Parameswaran et al., 2000). These signal transduction pathways have been studied in CGRP<sub>1</sub> receptor-transfected HEK 293 cells and in cells that endogenously express the CGRP<sub>1</sub> receptor (Aiyar et al., 1999; Disa et al., 2000; Parameswaran et al., 2000; Rorabaugh et al., 2001).

In contrast to the CGRP<sub>1</sub> receptor, the putative CGRP<sub>2</sub> receptor has not been cloned or well-characterized. The CGRP<sub>2</sub> receptor has only been identified by its low affinity (K<sub>B</sub> > 1 μM) for ho-CGRP<sub>8–37</sub> in functional studies in which a response to CGRP is measured (Dennis et al., 1990). Foulkes et al. (1991) and Waugh et al. (1999) reported that CGRP-induced dilation of porcine coronary arteries is blocked with low affinity (K<sub>B</sub> = 5 μM) by ho-CGRP<sub>8–37</sub>. In addition, the putative CGRP<sub>2</sub> receptor-selective agonist [Cys(ACM)<sup>2,7</sup>]ho-CGRP induces dilation of these arteries (Waugh et al., 1999). These studies establish that porcine coronary arteries have the prototypical characteristics of a tissue containing the putative CGRP<sub>2</sub> receptor.

In the present investigation, we used RT-PCR, radioligand binding, and data from previously reported isolated tissue experiments to compare the CGRP<sub>2</sub> receptor that is expressed in porcine coronary arteries with the porcine CGRP<sub>1</sub> receptor that has been previously cloned and expressed in HEK 293 cells. Ligand affinities in isolated tissue experiments (K<sub>B</sub>) and in radioligand binding experiments (K<sub>I</sub>) were also compared. We found that the CGRP<sub>2</sub> receptors that have been previously reported to mediate relaxation of isolated porcine coronary arteries are identified as CGRP<sub>1</sub> receptors by radioligand binding and RT-PCR. These data do not support the proposal that there are two CGRP receptor subtypes.

**Materials and Methods**

**Chemicals and Reagents.** Taq DNA polymerase, 10 times PCR buffer (200 mM Tris-HCl, pH 8.4, and 500 mM KCl), amplification grade DNase I, 100-base pair DNA ladder, TRizol, minimum essential media, fetal bovine serum, and antibiotic/antimycotic (containing 10,000 units/ml penicillin G, 10,000 μg/ml streptomycin sulfate, and 25 μg/ml amphotericin B) were purchased from Invitrogen (Carlsbad, CA). Moloney murine leukemia virus reverse transcriptase was purchased from PerkinElmer (Foster City, CA). The plasmid cloning vector was purchased from Invitrogen, and [125I]ho-CGRP was purchased from Amersham Pharmacia Biotech (Piscataway, NJ). Na<sub>2</sub>Ca-ethylenediaminetetraacetic acid, Tri(hydroxymethyl)amino methane, Sigmacote, and other chemicals were obtained from Sigma (St. Louis, MO). HEK 293 cells stably expressing the porcine CGRP<sub>1</sub> receptor were a generous gift from Dr. Allan R. Shatzman of SmithKline Beecham Pharmaceuticals (King of Prussia, PA).

**Peptide Synthesis.** Adrenomedullin, calcitonin, ho-CGRP<sub>8–37</sub>, ho-CGRP, and [Cys(ACM)<sup>2,7</sup>]ho-CGRP were purchased from Peninsula Laboratories (San Carlos, CA). All other peptides were synthesized by solid phase methods and purified by reversed phase high-performance liquid chromatography as previously described (Li et al., 1997; Smith and Hanly, 1997; Saha et al., 1998; Rist et al., 1999). The structure of these peptides was verified by amino acid analysis and electrospray ionization-mass spectrometry.

**RNA Isolation.** A fresh porcine heart was obtained from a local slaughterhouse and transported to the laboratory in ice-cold phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na<sub>2</sub>PO<sub>4</sub>, and 1.4 mM KH<sub>2</sub>PO<sub>4</sub>, pH = 7.3). The left circumflex coronary artery was isolated and cleaned of fat and connective tissue with the aide of a dissecting microscope, and the endothelium was removed by gentle scraping with a number 22 scalpel blade. The artery was wrapped in aluminum foil, frozen at −70°C, and pulverized with a hammer. TRizol reagent was used to isolate total RNA from approximately 100 mg of pulverized artery according to the manufacturer’s protocol. The RNA was dissolved in 20 μl of RNase-free water containing DNase I buffer and 5 units of amplification grade DNase I. The DNase I was removed by adding an equal volume of TRizol and repeating the RNA isolation procedure. The RNA was dissolved in RNase-free water and stored at −70°C.

**RT-PCR.** RT-PCR was used to identify CRLR mRNA in porcine coronary arteries with the primers shown in Fig. 1. These primers were designed based on the porcine CRLR complementary DNA (cDNA) sequence provided by Dr. Allan R. Shatzman of SmithKline Beecham Pharmaceuticals. CRLR mRNA was initially detected in porcine coronary artery using primers 2 and 4 (Fig. 1). cDNA was synthesized by reverse transcription in a 10-μl reaction volume containing 1 times PCR buffer, 3 μg of RNA, 5 mM MgCl<sub>2</sub>, 1 mM dNTP mixture, 25 pmol of antisense primer, and 25 units of Moloney murine leukemia virus reverse transcriptase. The reaction was in-
cubated in a PerkinElmer 2400 thermocycler at 42°C for 50 min followed by a 5-min incubation at 99°C. PCR was conducted in a 100-μl reaction volume containing 1 times PCR buffer, 3 mM MgCl₂, 0.2 mM dNTP mixture, 50 pmol of sense primer, 50 pmol of antisense primer, 10 μl of cDNA, and 2.5 units of Taq DNA polymerase. PCR conditions included an initial cDNA denaturation step at 95°C for 5 min followed by 20 cycles (95°C denaturation for 30 s, 55°C annealing for 30 s, and 72°C extension for 30 s) of PCR and a final extension period of 7 min at 72°C. Primers 2 and 5 (Fig. 1) were used to amplify the 3’ end of the CRLR mRNA coding region using the PCR conditions described above. Primers 1 and 3 (Fig. 1) were used to amplify the 5’ end of the CRLR mRNA coding region. PCR conditions for this pair of primers were the same as those described above except that the annealing temperature was changed to 50°C.

Sense (5’-GAC CAT CAG GAG CTA TAA AGA CC-3’) and antisense (5’-TGC CAG ACC ACC AGT GGC GTC-3’) primers were designed based upon the porcine RAMP 1 cDNA sequence (GenBank accession number AF312385). These primers were used to detect RAMP 1 mRNA in coronary arteries using the method described above. However, the annealing temperature was adjusted to 54°C, and 40 cycles of PCR were used. The products of all RT-PCR reactions were visualized on ethidium bromide-stained 1.5% agarose gels and subcloned into the pcR II vector. Both DNA strands of each RT-PCR product were sequenced using an Applied Biosystems 373 DNA sequencer (Foster City, CA). DNA sequences were analyzed using the Wisconsin Package version 10.1 (Genetics Computer Group, Madison, WI) software.

Cell Culture. HEK 293 cells stably expressing the porcine CGRP₁ receptor were grown in T-175 culture flasks in minimum essential medium that was supplemented with fetal bovine serum (10%), penicillin G (100 units/ml), streptomycin (100 μg/ml), and amphotericin B (0.25 μg/ml). The flask was placed in a humidified incubator in an atmosphere of 5% CO₂/95% air and maintained at 37°C. The cells were grown to confluence and then harvested for membrane preparations as described below.

Membrane Preparations. Culture media was removed from confluent cells, and the cells were rinsed three times with 25 ml of ice-cold phosphate-buffered saline. Cells were dislodged from the flask with a cell scraper in the presence of 10 ml of ice-cold phosphate-buffered saline and centrifuged at 4°C for 5 min at 1000g. The pellet was suspended in 25 ml of buffer A (50 mM Tris-HCl and 5 mM Na₂Ca-ethylene-diaminetetraacetic acid, pH 7.4) by vortexing and then homogenized with a glass-Teflon homogenizer. The homogenate was centrifuged at 100,000g for 30 min in a Beckman L5-50 ultracentrifuge. The pellet was washed twice by homogenization in 25 ml buffer B (50 mM Tris-HCl, 100 mM NaCl, and 5 mM MgCl₂, pH 7.4), followed by centrifugation at 4°C for 30 min at 100,000g. The supernatant was removed, and the dry pellet was stored for up to 1 month at 70°C. Protein content of the final pellet was determined by the method of Lowry et al. (1951).

Fresh pig hearts were obtained from a local slaughter house and transported to the laboratory in ice-cold phosphate-buffered saline. The left circumflex, right circumflex, and anterior descending coronary arteries were removed and cleaned of fat and connective tissue with the aid of a dissecting microscope. The outside diameter of all coronary arteries used in this investigation was >1 mm. The arteries were cut open, and the endothelium was removed by gentle scraping with a number 22 scalpel blade. The arteries were cut into small pieces with scissors and homogenized in 25 ml of buffer A with an Ultra-Turrax T25 tissue homogenizer for 3 min at 24,000 rpm. The homogenate was centrifuged at 4°C for 10 min at 1000g to remove particulate debris. The supernatant was centrifuged at 4°C for 30 min at 100,000g, and the resulting pellet was washed twice in buffer B as described above for membranes from HEK 293 cells. Protein content of the final pellet was determined by the method of Lowry et al. (1951). Membrane pellets were stored for up to 1 week at 70°C.

[125I]-hCGP Binding Kinetics. The association and dissociation rates of [125I]-hCGP binding to CGRP receptors were determined in membranes prepared from either porcine coronary arteries or from HEK 293 cells expressing the porcine CGRP₁ receptor. Frozen membrane pellets were rehomogenized in ice-cold binding buffer (50 mM Tris-HCl, 5 mM MgCl₂, 100 mM NaCl, 0.2% bovine serum albumin, and 0.1% bacitracin, pH 7.4) to a concentration of 50 to 100 μg of membrane protein/150 μl. Membrane protein homogenate (150 μl) was added to 13 to 100-mm glass test tubes pretreated with Sigmacote. Fifty microliters of ice-cold binding buffer was added to each test tube, followed by 50 μl of 200 μM [125I]-hCGP. To measure the association rate, tubes were quickly vortexed and incubated at 37°C for various times. Bound and free [125I]-hCGP were separated by vacuum filtration by pouring the tube contents through Whatman (Maidstone, UK) GF/B glass microfiber filters that were presoaked in 0.2% polyethyleneimine for 30 min. Each tube was rinsed three times with 5 ml of buffer B, and this buffer was also poured through the filter. To measure the dissociation rate, tubes were vortexed and incubated at 37°C for 30 min. Fifty microliters of 5 μM nonradiolabeled ho-CGRP was added to each tube, and the tubes were incubated at 37°C for various times. Bound and free [125I]-hCGP were separated by vacuum filtration as described above. The filters were carefully transferred into 12 x 75-mm polyethylene tubes and bound [125I]-hCGP was measured in a Wallac gammamaster 1277 (Gaithersburg, MD) γ-counter.

Competition Binding Assay. The radioligand binding assay used in this study has been previously described in detail (Abel et al., 1997). Frozen membrane pellets were rehomogenized in ice-cold binding buffer to a concentration of 50 to 100 μg of membrane protein/150 μl. Membrane protein (150 μl) homogenate was added to 13 x 100-mm glass test tubes pretreated with Sigmacote. The tubes were incubated in a 37°C shaking water bath for 50 min in the presence of 40 pM [125I]-hCGP and various concentrations of nonlabeled ligands. The total incubation volume was 250 μl. Non-specific binding was determined using 1 μM ho-CGRP. Whatman GF/B glass microfiber filters were soaked in 0.2% polyethyleneimine for 30 min prior to their use. Bound and free [125I]-hCGP were separated by trapping the membranes on the filters and washing with 15 ml of buffer B using a Brandel Model MG-48R cell harvester (Gaithersburg, MD). Bound [125I]-hCGP was measured as described above.

Data Analysis. Association and dissociation rates of [125I]-hCGP binding to CGRP receptors were determined by nonlinear regression analysis using the equations for exponential association and exponential decay. These calculations were performed using GraphPad Prism (San Diego, CA).

Three to five competition binding curves were performed in duplicate for each ligand. Specific binding was determined by subtracting nonspecific binding (defined using 1 μM ho-CGRP) from total binding, and the IC₅₀ for each competition curve was determined by nonlinear regression analysis using the GraphPad Prism. The data were fit to both a one-site and a two-site binding model, and the best fit model was determined using an F test. Hill slopes were calculated from nonlinear regression analysis using a sigmoid curve fit model. In kinetic studies, the K₁₅₀ of [125I]-hCGP was 40 pM in membranes prepared from HEK 293 cells expressing the CGRP₁ receptor and 14 pM in membranes prepared from porcine coronary arteries. Therefore, these values were used to convert IC₅₀ values to Kᵢ values by the Cheng-Prusoff equation. Mean pKᵢ values for each ligand were compared using a two-tailed Student’s t test to determine whether the pKᵢ values in HEK 293 cells were significantly different (p < 0.05) from the pKᵢ values in porcine coronary arteries.

Correlation plots were used to compare antagonist affinities (pKᵢ) determined by competition binding experiments with antagonist affinities determined by their ability to inhibit CGRP-induced relaxation of isolated coronary arteries (pKᵢ). GraphPad Prism was used to perform linear regression analysis, to determine the confidence interval of the correlation, and to calculate the slope and correlation coefficient of these data.
Results

Identification of CRLR and RAMP 1 mRNA in Porcine Coronary Arteries. Primers that spanned a 223-nucleotide segment of the CRLR mRNA (primers 2 and 4 in Fig. 1) were initially used to search for CRLR mRNA in porcine coronary artery. Porcine lung, the tissue from which this cDNA was originally cloned (Elshourbagy et al., 1998) was used as a positive control. A 223-base pair RT-PCR product was identified in both the porcine coronary artery and lung (Fig. 2A), and DNA sequence analysis demonstrated that this product encoded a portion of the porcine CRLR. To confirm that the entire coding region of the CRLR mRNA was present in the coronary artery, we used primers 1 and 3, and primers 2 and 5 (Fig. 1). RT-PCR products from each primer pair were subcloned and sequenced, and the entire nucleotide sequence was submitted to GenBank (GenBank accession number AF419317). The amino acid sequence encoded by this mRNA is identical to that previously reported by Elshourbagy et al. (1998). The coding sequence of this mRNA is 1389 nucleotides long and shares 92% and 85% sequence identity with its human and rat orthologs, respectively (accession numbers L76380 and X70658).

RAMP 1 is an accessory protein that is reportedly required for intracellular trafficking and maturation of the CRLR into the CGRP1 receptor (McLatchie et al., 1998). RAMP 1 mRNA has been previously identified in several human tissues and cell lines, including HEK 293 cells (McLatchie et al., 1998). However, RAMP 1 has not been previously identified in coronary arteries. Since antibodies for this protein were unavailable, we used RT-PCR to identify RAMP 1 mRNA in porcine coronary arteries with the primers described above (Fig. 2B). Porcine RAMP 1 shares 78%, 78%, and 82% nucleotide sequence identity with its rat, mouse, and human orthologs, respectively (GenBank accession numbers AJ001014, AF146522, and AF181550).

Kinetics of [125I]h-CGRP Binding to CGRP Receptors. The $K_d$ of [125I]h-CGRP was determined in membranes from porcine coronary arteries and from porcine CGRP1 receptor-transfected HEK 293 cells by independently measuring the association and dissociation rates of [125I]h-CGRP binding to CGRP receptors. The procedure for calculating $K_d$ values from kinetic experiments has been described in detail by Limbird (1996). [125I]h-CGRP dissociated from membranes prepared from porcine CGRP1 receptor-transfected HEK 293 cells and from porcine coronary arteries with dissociation rate constants of 0.38/min and 0.46/min, respectively. A representative dissociation curve is shown for CGRP1 receptor-transfected HEK 293 cells in Fig. 3A. The association rate constant of [125I]h-CGRP was also determined, and a representative association curve is shown in Fig. 3B. $K_{ass}$ values (0.76/min and 1.8/min in HEK 293 cells and coronary arteries, respectively) were converted to association rate constants (9.5 $\times$ 10^9/M/min and 3.4 $\times$ 10^10/M/min for CGRP1 receptor-transfected HEK 293 cells and coronary arteries, respectively) by the formula $K_{assoc} = (K_{obs} - K_{dissoc})/|\text{radioligand}|$. The calculated $K_d$ value ($K_{dissoc}/K_{assoc})$ was 40 pM in HEK 293 cells expressing the porcine CGRP1 receptor and 14 pM in coronary arteries. These values are similar to $K_d$ values previously reported for [125I]h-CGRP in saturation binding experiments using HEK 293 cells stably

Fig. 2. Identification of CRLR (A) and RAMP 1 (B) mRNA in porcine coronary artery by RT-PCR. Porcine lung was used as a positive control for the detection of CRLR mRNA. The inclusion/exclusion of reverse transcriptase in the reverse transcription reaction is indicated by ±RT. Marker bands are 200- and 300-base pair size markers, and arrows indicate the 223- and 236-base pair RT-PCR products obtained using primers specific for CRLR (A) and RAMP 1 (B), respectively.

Fig. 3. Representative dissociation (A) and association (B) curves for [125I]h-CGRP determined in HEK 293 cells expressing the porcine CGRP receptor. Similar curves were obtained in membranes prepared from porcine coronary arteries. The association equilibrium constant ($K_{assoc}$) was calculated by the equation: $K_{assoc} = (K_{obs} - K_{dissoc})/|\text{radioligand}|$. The binding equilibrium dissociation constant ($K_d$) was calculated by the equation: $K_d = K_{dissoc}/K_{assoc}$. 

CGRP1 Receptor in Porcine Coronary Arteries 1089
transfected with the porcine ($K_d = 38 \, \text{pM}$) or human ($K_d = 19 \, \text{pM}$) CGRP$_1$ receptor (Aiyar et al., 1996; Elshourbagy et al., 1998).

**Binding of CGRP Receptor Ligands to CGRP Receptors in HEK 293 Cells.** $^{[125]}$I-ho-CGRP was used to label CGRP receptors as previously described (Abel et al., 1997). Specific binding was >90% in membranes from CGRP$_2$ receptor-transfected HEK 293 cells, and maximal inhibition of $^{[125]}$I-ho-CGRP binding for each ligand (except calcitonin) was not different from the maximal inhibition caused by 1 $\mu$M ho-CGRP. ho-CGRP and the CGRP$_1$ receptor-selective ligand $\text{[Cys(ACM)$_{2,7}$]h-CGRP}_{8-37}$ bound with high affinity to membranes from HEK 293 cells stably expressing the CGRP$_1$ receptor (Fig. 4A; Table 1). The affinity of $\text{[Cys(ACM)$_{2,7}$]h-CGRP}_{8-37}$ was increased 4-fold by acetylation and 79-fold by benzoylation of the amino terminus. In contrast, the affinity of ho-CGRP$_{8-37}$ was dramatically decreased by replacing the phenylalanine at position 37 with either alanine ([Ala$_{37}$]ho-CGRP$_{8-37}$) or cyclohexylalanine ([Cha$_{37}$]ho-CGRP$_{8-37}$). [Pro$_{44}$]ho-CGRP, a putative CGRP$_2$ receptor-selective ligand, bound with relatively high affinity. The prototypical CGRP$_2$-selective agonist [Cys(ACM)$_{2,7}$]ho-CGRP bound to the CGRP$_1$ receptor with 400-fold lower affinity than ho-CGRP. Adrenomedullin, another member of the CGRP peptide family, competed for $^{[125]}$I-ho-CGRP binding sites with 70% in HEK 293 membranes and 90% in membranes from porcine coronary arteries with high affinity (Elshourbagy et al., 1998).

Previous studies have demonstrated that the affinity of ho-CGRP$_{8-37}$ was reduced by replacing the phenylalanine at position 37 with alanine or cyclohexylalanine ([Cha$_{37}$]ho-CGRP$_{8-37}$). Previous studies have demonstrated that the affinity of ho-CGRP$_{8-37}$ was increased 4-fold by acetylation and 79-fold by benzoylation of the amino terminus. In contrast, the affinity of ho-CGRP$_{8-37}$ was dramatically decreased by replacing the phenylalanine at position 37 with either alanine ([Ala$_{37}$]ho-CGRP$_{8-37}$) or cyclohexylalanine ([Cha$_{37}$]ho-CGRP$_{8-37}$). [Pro$_{44}$]ho-CGRP, a putative CGRP$_2$ receptor-selective ligand, bound with relatively high affinity. The prototypical CGRP$_2$-selective agonist [Cys(ACM)$_{2,7}$]ho-CGRP bound to the CGRP$_1$ receptor with 400-fold lower affinity than ho-CGRP. Adrenomedullin, another member of the CGRP peptide family, competed for $^{[125]}$I-ho-CGRP binding sites with 70% in HEK 293 membranes and 90% in membranes from porcine coronary arteries with high affinity (Elshourbagy et al., 1998).

Therefore, we characterized the CGRP receptors in coronary arteries with ligands that were predicted to bind with a broad range of affinities. ho-CGRP and ho-CGRP$_{8-37}$ bound to membranes from porcine coronary arteries with high affinity (0.11 and 6.6 nM, respectively). Consistent with previous studies (Smith et al., 2001), the affinity of ho-CGRP$_{8-37}$ was increased 5-fold by acetylation and 24-fold by benzoylation of the amino terminus. In contrast, the affinity of ho-CGRP$_{8-37}$ was reduced by replacing the phenylalanine at position 37 with alanine or cyclohexylalanine (Table 1). The CGRP$_2$ receptor-selective peptide [Cys(ACM)$_{2,7}$]ho-CGRP bound with a 349-fold lower affinity than ho-CGRP$_{8-37}$ and a 6-fold lower affinity compared with ho-CGRP$_{8-37}$. The high-affinity binding of [Cys(ACM)$_{2,7}$]ho-CGRP$_{8-37}$ and lower-affinity binding of [Cys(ACM)$_{2,7}$]ho-CGRP is consistent with the presence of the CGRP$_2$ receptor in this tissue. Adrenomedullin competed for $^{[125]}$I-ho-CGRP binding sites with 180-fold lower affinity than ho-CGRP, and calcitonin (1 nM–1 $\mu$M) did not compete for $^{[125]}$I-ho-CGRP binding sites at all. Competition binding curves for several of these ligands are shown in Fig. 4B, and mean $K_i$ values for all ligands are listed in Table 1. Competition curves for each ligand (except calcitonin) fit best to a single binding site model.

**Comparison of Ligand Affinities for CGRP Receptors in Porcine Coronary Arteries and HEK 293 Cells.** A comparison of ligand affinities in membranes from porcine coronary arteries and HEK 293 cells expressing the cloned porcine CGRP$_2$ receptor is shown in Table 1. There were no significant differences ($p < 0.05$) for any of these peptides when comparing their affinities for the CGRP receptors in porcine coronary arteries with their affinities for porcine CGRP$_1$
receptors transfected into HEK 293 cells. In addition, pKᵢ values in porcine coronary arteries and porcine CGRP₁ receptor-transfected HEK 293 cells demonstrated a strong correlation (r² = 0.99) (Fig. 5A).

Antagonist affinities (Kᵢ) determined by competition binding were 22- to 707-fold higher than the affinities that we have previously observed for these ligands in functional assays that measure their inhibition of CGRP-induced relaxation of isolated porcine coronary arteries (Table 2). Therefore, we examined the correlation between the affinity values determined by functional assays with coronary arteries (pKᵢ) and the affinities determined by competition binding using membranes (pKᵢ) from the same tissue. The affinity of each antagonist was higher when measured by radioligand binding than when measured in functional assays. Thus the linear regression lines correlating these data are not superimposed with the line of identity (Fig. 5 B). However, there was a high correlation (r² = 0.88) between affinity values determined by the two methods. There was also a high correlation (r² = 0.86) between radioligand binding affinities in CGRP₁ receptor-transfected HEK 293 cells and affinities determined by functional experiments with isolated coronary arteries (Fig. 5C). For both correlations (Fig. 5, B and C), the 95% confidence interval of the slope of the regression line included the value of 1.0.

**Discussion**

CGRP receptors have been classified into CGRP₁ and CGRP₂ receptor subtypes based upon their affinity for h-CGRP₈₋₃₇ in isolated tissue experiments. h-CGRP₈₋₃₇ is an antagonist that inhibits CGRP₁ receptor-mediated responses with high affinity (Kᵢ < 100 nM) and inhibits putative CGRP₂ receptor-mediated responses with low affinity (Kᵢ > 1 μM) (Dennis et al., 1990). In addition, [Cys(ACM)²,⁷]h-CGRP has been proposed to be an agonist at CGRP₂ receptors and inactive at CGRP₁ receptors (Dennis et al., 1989). In isolated tissue studies, Foulkes et al. (1991) have previously reported that CGRP receptors in large porcine coronary arteries (outside diameter >1 mm) have low affinity for h-CGRP₈₋₃₇ (Kᵢ > 1 μM). Our laboratory has also found that h-CGRP₈₋₃₇ has low affinity (Kᵢ > 1 μM) in large porcine coronary arteries and that [Cys(ACM)²,⁷]h-CGRP causes relaxation of this tissue (Waugh et al., 1999). These functional studies have established large coronary arteries as a model for studying the CGRP₂ receptor.

In contrast to previous studies, we report two independent lines of evidence to show that porcine coronary arteries express the CGRP₂ receptor. First, we have identified mRNA encoding CRLR and RAMP 1 in porcine coronary arteries. These proteins have been previously shown to form the CGRP₁ receptor (McLatchie et al., 1998). Second, we have found that CGRP₁ and CGRP₂ receptor-selective ligands do not discriminate between CGRP receptors in porcine coronary arteries and porcine CGRP₁ receptors that have been transfected into HEK 293 cells. Furthermore, correlations between affinity values determined by radioligand binding and by isolated tissue experiments suggest that the CGRP receptor that has low affinity for h-CGRP₈₋₃₇ in functional studies with coronary arteries is the same CGRP receptor that has been cloned and expressed in HEK 293 cells. These data do not support the current view that the CGRP₁ and CGRP₂ receptor subtypes are different proteins that represent independent receptors.

We used radioligand binding to compare the affinity of several ligands for CGRP receptors expressed in coronary arteries with their affinities for CGRP₁ receptors expressed in HEK 293 cells. Stable expression of the porcine CGRP₁ receptor in HEK 293 cells provides a system that is free of other receptors that might bind CGRP and its analogs. This also allowed the comparison of CGRP receptor subtypes from the same species. This is important because many other comparisons of CGRP₁ and CGRP₂ receptors have been complicated by the use of tissues from different species to represent the putative CGRP receptor subtypes. We found that the affinity of h-CGRP₈₋₃₇, the prototypical CGRP₁ receptor-selective antagonist, and of [Cys(ACM)²,⁷]h-CGRP, the putative CGRP₂ receptor-selective agonist, were nearly identical in membranes prepared from porcine coronary arteries and from porcine CGRP₁ receptor-transfected HEK 293 cells. In fact, each of the 12 ligands used in the radioligand binding

**TABLE 1**

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Pig Coronary Arteries</th>
<th>Transfected CGRP₁ Receptor</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Kᵢ</td>
<td>Hill Slopeᵃ</td>
</tr>
<tr>
<td>h-CGRP</td>
<td>0.11 ± 0.08</td>
<td>−0.94</td>
</tr>
<tr>
<td>[N-benzylo]h-CGRP₈₋₃₇</td>
<td>0.27ᵇ</td>
<td></td>
</tr>
<tr>
<td>[N-acetyl]h-CGRP₈₋₃₇</td>
<td>1.3 ± 0.8</td>
<td>−0.82</td>
</tr>
<tr>
<td>h-CGRP₈₋₃₇</td>
<td>6.6 ± 6.3</td>
<td>−0.90</td>
</tr>
<tr>
<td>[Pro³⁴]h-CGRP₈₋₃₇</td>
<td>9.0 ± 6.3</td>
<td>−0.70</td>
</tr>
<tr>
<td>[D-Pen²,⁷]h-CGRP₈₋₃₇</td>
<td>21.0 ± 5.9</td>
<td>−0.93</td>
</tr>
<tr>
<td>Adrenomedullin</td>
<td>19.0 ± 11.6</td>
<td>−0.97</td>
</tr>
<tr>
<td>[Cys(ACM)²,⁷]h-CGRP₈₋₃₇</td>
<td>38.4 ± 25.2</td>
<td>−0.81</td>
</tr>
<tr>
<td>[Cha⁷]h-CGRP₈₋₃₇</td>
<td>32.7 ± 13.6</td>
<td>−0.96</td>
</tr>
<tr>
<td>[Pro³⁴,Phe³⁵]h-CGRP²₇₋₃₇</td>
<td>50.7 ± 16.7</td>
<td>−1.05</td>
</tr>
<tr>
<td>[Ala³⁷]h-CGRP₈₋₃₇</td>
<td>499.9 ± 265</td>
<td>−1.00</td>
</tr>
<tr>
<td>Calcitonin</td>
<td>No binding</td>
<td></td>
</tr>
</tbody>
</table>

ᵃ The 95% confidence intervals of the Hill slope included the value of −1.0 for each ligand except for h-CGRP₈₋₃₇, [Pro³⁴]h-CGRP, [Ala³⁷]h-CGRP₈₋₃₇, and adrenomedullin in the h-CGRP₁ receptor clone.
ᵇ From Smith et al. (2001).
ligand binding in HEK 293 cells expressing the porcine CGRP1 receptor. C, the correlation between antagonist affinities determined by radioligand binding or isolated tissue experiments with porcine coronary arteries. B, the correlation between antagonist affinities determined by radioligand binding in porcine coronary arteries and HEK 293 cells expressing the porcine CGRP1 receptor. The solid line is the linear regression line calculated from the data points; the dotted lines represent the 95% confidence interval of the linear regression; and the dashed line represents the line of identity. The slope and the correlation coefficient ($r^2$) of the linear regression of the data points is also indicated.

### TABLE 2

Comparison of ligand affinities determined by competition binding ($K_i$) or isolated tissue experiments ($K_B$) in pig coronary arteries

<table>
<thead>
<tr>
<th>Antagonist</th>
<th>$K_i$</th>
<th>$K_B$</th>
<th>$K_B/K_i$</th>
</tr>
</thead>
<tbody>
<tr>
<td>[N-benzyol]h-CGRP$_{8-37}$</td>
<td>0.27$^a$</td>
<td>40.36$^d$</td>
<td>149</td>
</tr>
<tr>
<td>[N-acetyl]h-CGRP$_{8-37}$</td>
<td>1.3 ± 0.8</td>
<td>29.2$^d$</td>
<td>22</td>
</tr>
<tr>
<td>h-CGRP$_{8-37}$</td>
<td>6.6 ± 6.3</td>
<td>4,670$^d$</td>
<td>707</td>
</tr>
<tr>
<td>[D-Pen$_2$]h-CGRP</td>
<td>21.0 ± 5.9</td>
<td>629 ± 94.3$^d$</td>
<td>30</td>
</tr>
<tr>
<td>[Cha$<em>7$]h-CGRP$</em>{8-37}$</td>
<td>32.7 ± 13.6</td>
<td>9,528 ± 1,156$^d$</td>
<td>291</td>
</tr>
<tr>
<td>[Ala$<em>3$]h-CGRP$</em>{8-37}$</td>
<td>499.9 ± 265</td>
<td>205,589 ± 34,500$^d$</td>
<td>411</td>
</tr>
</tbody>
</table>

$^a$ Smith et al. (2001).  
$^b$ Waugh et al. (1999).  
$^c$ Saha et al. (1998).  
$^d$ D. J. J. Waugh, personal communication.

Our radioligand binding data raise an important question: Why does h-CGRP$_{8-37}$ appear to identify a low-affinity CGRP$_1$ receptor in functional assays with isolated tissue but not in competition binding experiments? One explanation for the low affinity of h-CGRP$_{8-37}$ in isolated porcine coronary arteries, rat vas deferens, and other tissues is that this ligand may be degraded by proteases, causing the peptide to appear to have a lower affinity in these tissues than in tissues that lack these enzymes. Fernandez-Patron et al. (2000) reported that matrix metalloprotease-2, a protease present in vascular smooth muscle and endothelium, specifically cleaves h$_2$-CGRP into h$_2$-CGRP$_{1-14}$ and h$_2$-CGRP$_{15-37}$. In addition, we found that acetylation of the amino terminus of h$_2$-CGRP$_{8-37}$, a modification that has been demonstrated to protect other peptides from degradation (Drapeau et al., 1993), caused a 160-fold increase in the affinity ($K_B$) of this ligand in functional relaxation assays with porcine coronary arteries (Smith et al., 2001). In
contrast, acetylation of hCGRP8–37 caused only a 5-fold increase in its binding affinity ($K_I$) for membranes from the same tissue. These data suggest that hCGRP8–37 may be more susceptible to proteolytic degradation in whole coronary arteries than in membranes. Two nonpeptide CGRP receptor ligands (SB-273779 and BIBN4096BS) have recently been developed (Doods et al., 2000; Aiyar et al., 2001) and may be useful for avoiding ligand degradation while studying CGRP receptors.

A disadvantage of radioligand binding experiments using membranes is that the receptor is removed from its native environment and placed under conditions that may cause receptor accessory proteins to be lost. We found that hCGRP8–37 has a low, CGRP2 receptor-like affinity in functional studies of isolated porcine coronary arteries and a high, CGRP1 receptor-like affinity in membrane binding assays. One explanation for this difference is that the binding characteristics of the CGRP1 receptor are modified during the membrane preparation procedure. McLatchie et al. (1998) and Evans et al. (2000) have reported that RAMP 1 and receptor component protein (RCP) are required to form a functional CGRP1 receptor. RAMP 1 is an integral membrane protein with a membrane-spanning domain that presumably protects it from being lost during the membrane preparation procedure. However, RCP is a peripheral membrane protein that can be dissociated from the membrane (Evans et al., 2000). Although the affinity of hCGRP is unaffected by the presence or absence of RCP (Evans et al., 2000), the effect of this protein on hCGRP8–37 has not been examined. It is possible that low-affinity hCGRP8–37 binding is conferred by RCP in intact tissues and that hCGRP8–37 does not have low affinity in competition binding experiments because RCP is lost during the membrane preparation procedure.

The binding affinities of CGRP receptor agonists were also determined in our study. Previous investigators have reported that [Cys(ACM)2,7]hCGRP and [Pro14]hCGRP are selective for the putative CGRP2 receptor (Dennis et al., 1989; Li et al., 1997). In contrast, [Cys(ACM)2,7]hCGRP and [Pro14]hCGRP demonstrated no selectivity for CGRP receptors in coronary arteries over CGRP1 receptors in HEK 293 cells in our competition binding experiments. Furthermore, we have found that the putative CGRP2 receptor agonist, [Pro14]hCGRP, stimulates 3',5'-cyclic adenosine monophosphate production ($EC_{50} = 158.7 \pm 113.2 \text{nM}$) in porcine CGRP receptor-transfected HEK 293 cells (B. R. Rorabaugh, P. W. Abel, D. D. Smith, and M. S. Soffield, unpublished data). The ability of these ligands to demonstrate agonist activity in some CGRP1 receptor systems and not in others suggests that the selectivity of these ligands is caused by something other than the presence of a second CGRP receptor subtype. One possibility is that the tissue to tissue variation in potency of these agonists is caused by different amounts of receptor reserve. We have previously shown that [Cys(ACM)2,7]hCGRP is a partial agonist in porcine coronary arteries (Waugh et al., 1999). This is consistent with the presence of a CGRP1 receptor reserve in tissues in which [Cys(ACM)2,7]hCGRP is an agonist and an absence of a CGRP2 receptor reserve in tissues in which this ligand shows no agonist activity (Kenakin, 1993). Therefore, the existence of a second CGRP receptor subtype is not necessary to explain the ability of [Cys(ACM)2,7]hCGRP and [Pro14]hCGRP to be agonists in some isolated tissues and inactive in others.

In summary, CGRP receptors in porcine coronary arteries have been previously classified as the CGRP2 receptor subtype because hCGRP8–37 antagonizes CGRP-induced relaxation of these arteries with low affinity. However, we have demonstrated that porcine coronary arteries have CRLR and RAMP 1 mRNA and that the ligand binding characteristics of CGRP receptors in porcine coronary arteries are identical to those of the cloned porcine CGRP1 receptor. Furthermore, the correlation between $K_I$ and $K_t$ values are consistent with the conclusion that the low affinity ($K_I > 1 \mu \text{M}$) of hCGRP8–37 in functional studies using isolated porcine coronary arteries occurs at the CGRP2 receptor. Our data do not support the idea that CGRP1 and CGRP2 receptors represent two independent proteins with different affinities for the antagonist, hCGRP8–37. Rather, our results suggest that there is only one CGRP receptor that can have different affinities for hCGRP8–37 in functional studies, based upon various tissue-dependent factors.

Acknowledgments

We thank Joe Haun of J & J Quality Meats (Elkhorn, NE) and Al Lieberum of Hormel Foods Corporation (Fremont, NE) for providing porcine tissues. HEK 293 cells stably expressing the porcine CGRP1 receptor were a gift from Dr. Allan R. Shatzman at SmithKline Beecham.

References


Address correspondence to: Dr. Peter W. Abel, Department of Pharmacology, Creighton University School of Medicine, 2500 California Plaza, Omaha, NE 68178. E-mail: pabel@creighton.edu