A cDNA Encoding the Calcitonin Gene-related Peptide Type 1 Receptor*

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Calcitonin gene-related peptide (CGRP) is a neuropeptide with diverse biological effects including potent vasodilator activity. We report here the cloning of a complementary DNA (cDNA) encoding a human CGRP1 receptor, which shares significant peptide sequence homology with the human calcitonin receptor, a member of the G-protein-coupled receptor superfamily. Northern blot analysis revealed that the messenger RNA for this receptor is predominantly expressed in the lung and heart. In situ studies showed specific localization of the receptor mRNA to alveolar cells in the lung and to cardiac myocytes in the heart. Stable expression of the CGRP receptor was used as a probe to screen an oligo(dT)-primed human lung cDNA library. Exposure of these cells to CGRP resulted in a 60-fold increase in cAMP production, which was inhibited in a competitive manner by the CGRP1 receptor antagonist, CGRP-(8–37).

Calcitonin gene-related peptide (CGRP)1 is 37-amino acid peptide that exists as highly homologous α or β isoforms in both human and rat (1, 2). α- and β-CGRP display very similar biological activities, including peripheral and cerebral vasodilation (3), cardiac acceleration (4), regulation of calcium metabolism (5), reduction of intestinal motility (6), regulation of glucose metabolism (reduction of insulin secretion and insulin sensitivity) (7), diminution of appetite (8), and reduction of growth hormone release (9). The two CGRP peptides differ by 3 amino acids in humans and 1 amino acid in rats. The amino acid sequences of CGRP peptides are well conserved among species and can be considered as members of a family of peptides including the related peptides amylin (46% homology), salmon calcitonin (32% homology), and adrenomedullin (24% homology). These peptides in general have N-terminal ring structures of 6–7 amino acids involving a disulfide bridge and an amidated C-terminal end (10–12).

CGRP peptides are localized predominantly in sensory afferent nerves and central neurons (11, 12). When released from the cell, the peptides initiate their biological responses by binding to specific cell surface receptors, which are predominantly coupled to the activation of adenyl cyclase (3, 13). CGRP receptors have been identified and pharmacologically evaluated in several tissues, including brain, cardiovascular, endothelial, and smooth muscle tissues (12). Multiple CGRP receptors have been observed, based on pharmacological properties they are divided into at least two subtypes and denoted as CGRP1 and CGRP2, according to the classification of Dennis et al. (14). CGRP1-(8–37), which lacks 7 N-terminal amino acid residues, is a selective antagonist of CGRP1 receptors, whereas the linear analog of CGRP, diacetoamidomethyl cysteine CGRP (Cys(ACM2,7)CGRP), is a selective agonist of CGRP2 receptors (14).

A deeper understanding of the physiological and pathophysiological effects mediated by the CGRP peptides has been constrained for lack of a cloned cognate receptor. Here we describe the isolation by expressed sequence tag (EST) analysis (15–17) of a human CGRP receptor that exhibits ligand binding and functional properties of the CGRP1 receptor. Curiously, this receptor had been cloned previously as an orphan calcitonin-like receptor receptor (rat 18) and, recently, human (19), but the authors had been unable to demonstrate an interaction with CGRP or any other ligands.

EXPERIMENTAL PROCEDURES

Materials—[2-125I]iodohistidyl[29]Human (h) CGRPα (specific activity 2000 Ci/mmol) was obtained from Amersham. hCGRPα, hCGRPα-(8–37), salmon calcitonin (CT), human CT, porcine vasoactive intestinal peptide, and angiotensin II were purchased from Bachem Biochemicals. Cys(ACM2,7)CGRP was obtained from Phoenix Pharmaceuticals (Belmont, CA). Salmon CT-(8–32) was synthesized at SmithKline Beecham Pharmaceuticals. BCA protein assay kit was obtained from Pierce. All other reagents were obtained from Sigma.

cDNA Cloning—Expressed sequence tag (EST) analysis (15–17) of cDNA clones derived from a human synovial tissue cDNA library (digoDT-primed and constructed in the λ ZAPII vector (Stratagene)) identified a 800-bp clone demonstrating significant homology to the human CT receptor. This cDNA clone encoding an incomplete hCGRP1 receptor was used as a probe to screen a digoxigenin-primed human lung cDNA library constructed in the λ ZAPII vector (Stratagene). cDNA library construction and screening were carried out essentially as described (20). Several positive clones were obtained, the longest of which was sequenced to completion by a ABI sequencer (13) (GenBank™ accession number L76380).

Stable Expression in HEK 293 Cells—The CGRP receptor has three potential in-frame ATG start codons; however, the most 3′prime-ATG codon resides in the most favorable Kozak consensus context. For this reason, and to potentially increase protein translation efficiency (21), we prepared a 1.4-kilobase cDNA fragment by PCR amplification that
encompassed the entire CGRP receptor coding region, beginning with the 3'-most ATG codon, and subcloned this fragment into the mammalian expression vector, pcDN (22). The oligonucleotide primers used for PCR amplification were 5'-GGG TAC CCC ACC ATG AGG AAA TGT ACC TCG TAT TTT CTG G-3' and 5'-GGG GAT CCC GCA AAC AGT GAG ACA ACC ATC CTG TTA TGA AT-3' (the translation start and stop codons are underlined). Human HEK 293 cells were grown in 100-mm culture dishes and transfected with 10 μg of the CDN-CGRP-receptor cDNA using the LipofectAMINE transfection reagent (Life Technologies, Inc.) according to the manufacturer's instructions. After 2 weeks of G418 selection (0.8 mg/ml), colonies were picked and expanded. A single cell line (among many potential candidates) that expressed a CGRP receptor protein (by the binding assay) (cAM) was chosen for further analysis. A single cell line transfected with pcDN vector alone was selected for use as a control for functional and binding assays.

Northern Blot Analysis—Total RNA was isolated from human tissues by TriZOL Reagent (Life Technologies, Inc.). 20 μg of total RNA from each tissue was separated by formaldehyde, 1.0% agarose gel electrophoresis and transferred to a nylon membrane. The full-length human CGRP cDNA was labeled and used as a probe. Hybridization was done essentially as described (23).

In Situ Gene Amplification Analysis—Under anesthesia, a BALB/C mouse was perfused with 4% paraformaldehyde (pH 7.5). Organs were dissected, fixed in 10% buffered formalin at room temperature for 3 days, then embedded in paraffin. Sections of 5 μm were prepared. Reverse transcriptase in situ gene amplification was performed as described (24). Two primers used in this study were derived from gene amplification were detected by anti-digoxigenin antibody conjugated to alkaline phosphatase (Genius3, Boehringer Mannheim).

Functional Assays—Transformed or untransformed 293 cells were plated at 2.5 × 10⁴ cells/well in 6-well plates. On day 4, the medium was aspirated and the cells were washed with 1 ml of Dulbecco's phosphate-buffered saline (pH 7.4) containing 0.5 mM isobutylmethylxanthine for 10 min at room temperature. The cells were treated with various concentrations (1 pM to 1 μM) of CGRP or related peptides at 37°C for 10 min. The reaction was stopped by addition of 100 μl of 100% ice-cold trichloroacetic acid to each well, and cAMP in each well was measured following the radiomunnoassay protocol as described (Advance Magnetics). Each experiment was performed in triplicate and repeated 2-3 times with different passages of cells.

RESULTS AND DISCUSSION

We hypothesized that the CGRP and recently cloned calcitonin (CT) receptors might display close homology since (i) like the G-protein-coupled CT receptor (26), CGRP signaling appears to be mediated through the activation of adenyl cyclase, (ii) at high concentrations CT interacts with the CGRP binding site (27), and (iii) CGRP (the α, not β, isopeptide) and CT share the same mRNA transcript and are produced through alternate splicing (1), as are the substance P/K ligands that cross-react with tachykinin receptors (28). Thus, when expressed sequence tag (EST) analysis (15–17) of cDNA clones derived from a human synovial tissue cDNA library identified a clone demonstrating significant homology to the human CT receptor, this cDNA was selected for further evaluation. Since the cDNA insert was incomplete (i.e. about 800 bp), it was used as a probe to screen a human lung cDNA library to isolate a clone with a complete open reading frame.

Fig. 1 shows the 2995-bp nucleotide sequence and deduced amino acid sequence of the human CGRPceptors. Amino acids (represented by the one-letter code) are indicated below their respective codons and numbered 1 at the left beginning with the ATG initiation methionine codon (M). Other numbers on the right indicate nucleotide positions starting at position 1 of the A nucleotide of the ATG codon.

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rat CT-like "orphan" receptor, with which it shares 91% amino acid sequence homology and thus likely represents a receptor ortholog (18). Also, since the third ATG most closely approximates a Kozak consensus translation initiation site (29), it is probably the translation initiation codon. Consequently, the cDNA encodes a protein of 461 amino acids, sharing several features in common with the G-protein-linked receptors (30, 31). Most prominent is the existence of seven hydrophobic regions of 16 to 28 amino acids each, which are likely to be membrane-spanning domains that form a seven-transmembrane motif found among G-protein-coupled receptors. In addition, 52 amino acid residues highly conserved among a recently described subfamily of G-protein-coupled receptors including calcitonin (CT), secretin, parathyroid, glucagon, and other receptors (20, 26, 32–34), are also present, interspersed within the sequence. Among this subfamily, the doned protein shares its greatest sequence identity, 55.5%, with the human CT receptor. Furthermore, within the N-terminal domain there are several sites for post-translational modification including three asparagine residues within consensus sites for glycosylation and a potential cleavage site of an N-terminal hydrophobic sequence that may be a signal peptide.

Northern blot analysis of the full-length cDNA as a hybridization probe revealed mRNA species of approximately 7.5, 5.5, and 3.5 kb predominantly in the lung and to a lesser degree in the heart (Fig. 2A). RNA dot-blot analysis of over 20 different human tissues indicated widespread, but generally low levels, of receptor mRNA expression (data not shown). In situ analysis of mouse lung and heart tissues demonstrated specific expression of the CGRP₁ receptor mRNA in alveolar cells in the lung and cardiac myocytes in the heart (Fig. 2B).

**Fig. 2. Tissue expression of CGRP₁ receptor mRNA.** A, Northern blot analysis of CGRP₁ mRNA expression. RNA size markers are indicated on the left. B, in situ gene amplification analysis of CGRP₁ mRNA expression. The adjacent sections of mouse lung (a, b, c, d, × 100) and heart (e, f, g, h, × 400) were used for these studies. a and e, use of the reverse transcriptase. b and f, omission of the reverse transcriptase. c and g, omission of amplification primers. d and h, hematoxylin and eosin staining.

**Fig. 3. Pharmacological characterization of the recombinant CGRP receptor stably expressed in 293 cells.** A, representative saturation curve for ¹²⁵I-CGRP binding to the membranes prepared from 293 cells expressing the recombinant CGRP receptor. ▲, total binding; ●, nonspecific binding; and ○, specific binding. Inset, corresponding Scatchard plot of the data. B, competition binding profile of CGRP (▲) and its analogs CGRP-(8–37) (●), Cys(ACM2,7)CGRP (○), ADM (◇), salmon CT (●), salmon CT-(8–32) (○), human CT (□), angiotensin II (◇), and vasoactive intestinal peptide (◇) for specific ¹²⁵I-CGRP binding to the membranes. C, competition binding profile of CGRP and its analogs for specific ¹²⁵I-CGRP binding to SK-N-MC cell membranes.
To determine the binding and functional properties of the receptor, we used HEK 293 cells stably transfected with the cdNA subcloned within an expression vector. Membranes prepared from untransfected 293 cells had very little specific 125I-CGRP binding (data not shown). Membranes prepared from transfected 293 cells displayed high-affinity, low-density binding sites for 125I-CGRP. The apparent dissociation constant (K_d) and maximum binding (B_max) were 19 ± 3 pm and 86 ± 14 fmol/mg protein, respectively (Fig. 3A). In competition binding studies, the rank order potency for a series of related peptides to inhibit 125I-CGRP binding to these membranes was CGRP > CGRP-(8–37) > Cys(ACM2,7)CGRP > adrenomedullin (ADM) > salmon CT-(8–32) >> salmon CT > human CT > vasoactive intestinal peptide > angiotensin II (Fig. 3B). The binding affinity of 125I-CGRP for the recombinant receptor as well as the pharmacological profile of the competing ligands were very similar to that observed for endogenous CGRP receptors present in membranes prepared from human neuroblastoma SK-N-MC cells (Fig. 3C).

Previous studies have shown that CGRP mediates its responses by activation of adenylyl cyclase and the generation of cyclic AMP (4, 5). Treatment of vector-transfected 293 cells with CGRP induced less than a 2-fold increase in the accumulation of cAMP (Fig. 4A). Addition of CGRP to 293 cells expressing the recombinant receptor resulted in an increased accumulation of cAMP that was concentration-dependent (Fig. 4B). The threshold, half-maximal, and maximal concentrations of CGRP required to stimulate cAMP accumulation in these cells were 0.1, 0.9, and 10 nM, respectively. The maximal stimulation in response to agonist was 60-fold over basal levels of cAMP. Again, these results were comparable to those observed using SK-N-MC cells containing endogenous CGRP receptors (35). The availability of the cDNA-transfected 293 cells with increasing concentrations of CGRP-(8–37), a selective CGRP receptor antagonist, shifted the CGRP concentration response curve for cAMP accumulation to the right in a parallel manner, indicating competitive inhibition with a calculated pA2 value of 7.57 (Fig. 4C). Except at high concentrations (1 μM), the CGRP1 receptor-selective agonist Cys(ACM2,7)CGRP failed to stimulate cAMP accumulation in both receptor-transfected cells (Fig. 4B) and SK-N-MC cells (data not shown). The effect of Cys(ACM2,7)CGRP on the recombinant CGRP receptor in inhibiting only 125I-CGRP binding but not a functional cAMP response to CGRP is consistent with the properties of the native rat CGRP1 receptor present in spleen membranes (36). Thus, the receptor described in the present study is a CGRP1 receptor.

At high concentrations, ADM evoked a specific response (Fig. 4B), which was not surprising since this agonist has been reported to interact weakly with the CGRP receptor (37). In contrast, human CT does not interact with the recombinant receptor, since this ligand stimulated a nearly identical activity in both CGRP receptor- and vector control-transfected cells (Fig. 4, A and B). Collectively, the pharmacological and functional results confirm that the receptor cdNA encodes a human CGRP1 receptor that is functionally coupled to the activation of adenylyl cyclase.

Although originally identified as an orphan rat CT-like receptor (18) and, very recently, classified as an orphan human CT-like receptor (despite testing for interaction with CGRP in addition to other ligands (19)), the results presented in this report clearly demonstrate that we have cloned a CGRP1 receptor. As to the reason why previous investigators were unable to identify the reported CT-like receptor as the CGRP1 receptor is only a matter of speculation. It is possible that the recombinant CGRP receptor can be functionally expressed only in certain cell types. In this study we used HEK 293 cells, whereas in other reports it appears that only COS and OK cells were used for receptor evaluation. We have recently cloned the porcine CGRP1 receptor ortholog of the human receptor and found it to exhibit essentially identical pharmacological properties, which provides further supporting evidence that we have identified the CGRP1 receptor (2). The availability of the CGRP receptor should facilitate the study of the physiology and pathophysiology of CGRP as a neurotransmitter, neuromodulator, local hormone, and inflammatory mediator.

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