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Cloning and Expression of an ATP-Regulated Human Retina C-Type Natriuretic Factor Receptor Guanylate Cyclase†

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ABSTRACT: The natriuretic factors are structurally related polypeptide hormones that regulate the hemodynamics of the physiological processes of diuresis, water balance, and blood pressure. Presumably, these hormones act through the activation of guanylate cyclases which are also the specific receptors of these hormones. Two such structurally similar cell surface receptors are known; the ligand for one is atrial natriuretic factor (ANF) and for the other is C-type natriuretic peptide (CNP). Studies with ANF receptor guanylate cyclase (ANF-RGC) have indicated that its ligand binding site is extracellular and the catalytic site is intracellular, but the mere ligand binding to the receptor domain does not activate the cytosolic catalytic domain. An intervening ATP-mediated event is obligatory: ATP binds to a defined ATP-regulated module (ARM) sequence and bridges the events of ligand binding and signal transduction. The mechanism of CNP signaling is not known, although CNP in intact cells transfected with CNP receptor guanylate cyclase (CNP-RGC) stimulates the formation of cyclic GMP. Furthermore, there is no prior evidence of the presence of CNP signal transduction system in retina, although the presence of ANF-RGC has been documented. We now report the molecular cloning and expression of CNP-RGC from human retina and show that ATP is obligatory in CNP signaling also. Compared to the previously published sequence of CNP-RGC cDNA isolated from human brain, the sequence of retinal cDNA reveals three new structural aspects at the noncoding regions: (1) it contains an additional stretch of 367 nucleotides at the 5′ region; (2) this stretch is followed by a 37-nucleotide sequence that is different from the corresponding brain sequence; and (3) the 3′ region contains an additional sequence of 20 nucleotides. The presence of signal transducers for both ANF and CNP peptides in retina suggests that these peptides, in addition to their traditional roles as regulators of the hemodynamic processes of cardiovasculature, have additional modulatory roles in the neural retina also.

Since the original discovery of a natriuretic factor (deBold, 1982, 1985; deBold et al., 1981), termed ANF, that regulates the hemodynamics of the physiological processes of diuresis, water balance, and blood pressure, two other structurally related natriuretic factors—brain natriuretic peptide (BNP) and C-type natriuretic peptide (CNP)—have been characterized [reviewed in Brenner et al. (1990), Needleman et al. (1989) and Rosenzweig and Seidman (1991)]. One important second messenger of all these three hormones is cyclic GMP (Brenner et al., 1990; Needleman et al., 1989; Rosenzweig & Seidman, 1991). The receptors for ANF (ANF-RGC)

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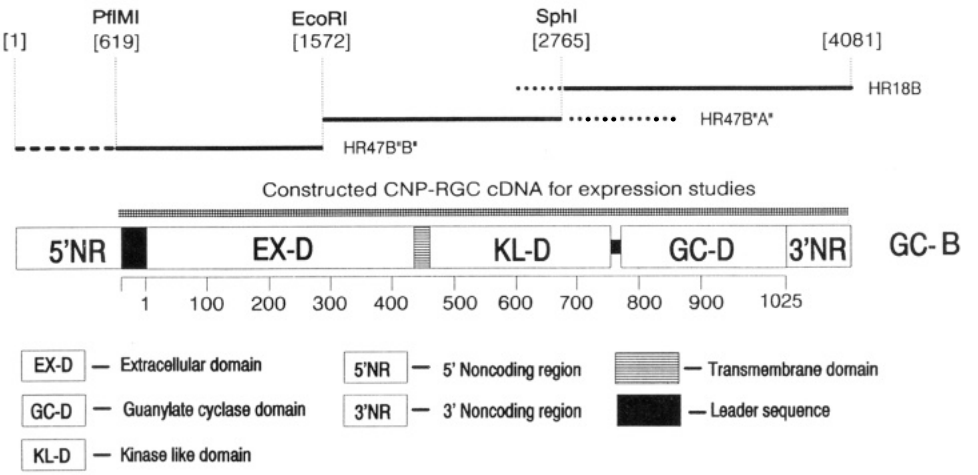

FIGURE 1: Construction of human retinal CNP-RGC. Overlapping sequences of two clones [18B and 47B (subclones 47B"A" and 47B"B")] from the human retinal cDNA library were used to construct the full-length CNP-RGC cDNA as described under Experimental Procedures. The bold lines represent those clone fragments which were ligated, the dotted lines represent the overlapping regions, the dashed line represents the 5′ noncoding region, and the shaded line represents the constructed CNP-RGC cDNA. Restriction sites used in the construction of CNP-RGC cDNA are in boldface letters; positions of these restriction-sites are indicated in brackets. The predicted topography of the CNP-RGC is represented as a box.

(Chinkers et al., 1989; Duda et al., 1991; Kuno et al., 1986; Marala et al., 1992; Meloche et al., 1988; Takayanagi et al., 1987) and CNP (CNP-RGC) (Koller et al., 1991) have been characterized; both are guanylate cyclases and are structurally similar. Topographical models of these receptor proteins indicate similarity in that both contain a single membrane-spanning helical domain which divides the protein into two roughly equal portions, the N-terminal extracellular and the C-terminal intracellular; the receptor domain lies in the extracellular portion while the intracellular portion contains two domains; the one adjacent to the transmembrane is termed "kinase-like" domain due to its sequence similarity to the tyrosine kinase family, and the C-terminal region contains the catalytic domain (Chinkers & Garbers, 1989). This topographical arrangement for the ANF-RGC protein is supported by site-directed (Goraczniak et al., 1992; Marala et al., 1992) and deletion-mutagenesis studies (Chinkers & Garbers, 1989; Goraczniak et al., 1992), in which the truncated-receptor cyclase showed no ANF-binding and ANF-dependent cyclase activities.

Recent studies with ANF-RGC show that the mere binding of ANF to the receptor domain is not enough to stimulate the cyclase activity and that ATP is obligatory for ANF signaling (Chinkers et al., 1991; Marala et al., 1991). ATP binding to a site on guanylate cyclase, termed ATP-regulated module (ARM) (Goraczniak et al., 1992; Marala et al., 1992), causes allosteric change in guanylate cyclase, bringing it to the activated state. The mechanism of CNP-RGC-mediated signal transduction is not known.

At present the consensus is that ANF signaling systems exist primarily in the periphery where they regulate cardiovasculature-related hemodynamic processes (Brenner et al., 1990; Needleman et al., 1989; Rosenzweig & Seidman, 1991). But this may not be the sole target site and function of ANF-like peptides and specifically of CNP. Retina is a neural tissue, and in this tissue the presence of ANF-RGC has been well documented immunologically (Cooper et al., 1989) and by molecular cloning studies (Barnstable & Ahmad, 1992; Duda et al., 1992; Kutty et al., 1992). Furthermore, preliminary studies involving Northern-blot analysis suggest that CNP is primarily localized in the central nervous system or in the cells of neural crest origin (Kojima et al., 1990; Sudoh et al., 1990). Direct molecular cloning studies have established the presence of CNP-RGC in brain (Chang et al., 1990). The presence of both CNP and its transducer, CNP-RGC, in the central nervous system supports the hypothesis that CNP has a neuromodulatory role (Kojima et al., 1990; Sudoh et al., 1990). If this hypothesis is correct, then there is a high probability that the retina, being a neural tissue, also contains a CNP signaling system along with the ANF signaling system. But, to date, attempts to demonstrate CNP-RGC in retina have failed (Barnstable & Ahmad, 1992).

In the present study, we directly establish the presence of a CNP-RGC in human retina by molecular cloning/expression studies and for the first time demonstrate that ATP is obligatory in CNP signaling. These results support the neuromodulatory role of CNP in human retina.

EXPERIMENTAL PROCEDURES

Materials. The 5-kDa CNP used in these studies was a 45 amino acid peptide, Ser-Gln-Asp-Ser-Ala-Phe-Arg-Ile-Gln-Glu-Arg-Leu-Arg-Asn-Ser-Lys-Met-Ala-His-Ser-Ser-Ser-Cys-Phe-Gly-Gln-Lys-Ile-Asp-Arg-Ile-Gly-Ala-Val-Ser-Arg-Leu-Gly-Cys-Asp-Gly-Leu-Arg-Leu-Phe; and ANF (rat, residues 8–33) was a 26 amino acid peptide, Arg-Arg-Ser-Ser-Cys-Phe-Gly-Gly-Arg-Ile-Asp-Arg-Ile-Gly-Ala-Gln-Ser-Gly-Leu-Gly-Cys-Asn-Ser-Phe-Arg-Tyr. These peptides were purchased from Peninsula Laboratories; GTP, cyclic GMP, and bovine serum albumin were from Sigma; ATPγS and ATP were from Boehringer-Mannheim; [^{125}I]NaI was from Amersham; cell culture media were from Gibco; and the restriction enzymes were purchased from USB and BioLabs.

Molecular Cloning. The isolation of a full-length CNP-RGC cDNA clone was the result of three successive screenings of a human retina cDNA library. Two partial clones with overlapping regions were used to construct the full-length cDNA. This cloning procedure is schematically represented in Figure 1 and is detailed below.

A phage cDNA library constructed from human retina poly-(A)$^+$ RNA and cloned into the *Eco*R1 site of the λgt10 vector (Clontech) was first screened with a probe corresponding to the nucleotide sequence 2930–3109 of GCα cDNA (Duda et al., 1991). This probe was constructed by polymerase chain reaction (PCR). The cDNA library was plated at a density of 50 000 plaques per plate; plaques were transferred to nitrocellulose filters, treated by standard procedures (Benton

& Davis, 1977), prehybridized for 1 h, at 37 °C in a buffer consisting of 0.02 M Tris-HCl, pH 7.5, 5× SSC (1× SSC contains 0.15 M NaCl, 15 mM trisodium citrate, pH 7.0), 1× Denhardt's solution [1× Denhardt's contains, per 500 mL, 0.1 g of Ficoll 400, 0.1 g of polyvinylpyrrolidone, and 0.1 g of bovine serum albumin (Pentax Fraction V)], 0.28 μg/mL sheared salmon sperm DNA and 50% deionized formamide, and hybridized to randomly labeled probe (Feinberg & Vogelstein, 1984) at 42 °C for 18 h in the same buffer as used in prehybridization. Filters were washed in 2× SSC plus 0.1% sodium dodecyl sulfate (SDS) at room temperature for 30 min and subsequently in 0.25× SSC plus 0.1% SDS at 60 °C for 30 min. XAR5 (Kodak) film was exposed to filters overnight at room temperature. Eight positive clones were subcloned into the *Eco*R1 site of pBluescript vector and sequenced by the dideoxy chain termination method (Sanger et al., 1977) on denatured double-stranded plasmid templates using Sequenase 2.0 (USB). Sequence analysis revealed that all eight clones were partial, four corresponded to the human ANF-RGC and four to the CNP-RGC. The longest (1.6 kb) clone (18B) matched the corresponding nucleotide sequence of fragment 2160-poly(A)$^+$ of human brain CNP-RGC (Chang et al., 1989) and was then used as a probe in the second screening of the phage cDNA library. This resulted in the isolation of five positive clones. One of them was a partial 1.5 kb clone (30B), and its sequence matched the brain CNP-RGC nucleotide region 1549–3021 (Chang et al., 1989). The 555 bp 5′ fragment of this DNA, corresponding to the nucleotide sequence region 1549–2104, was used as a probe in the third screening of cDNA library. This resulted in isolation of the 21 positive clones; the largest of the lot was a 3.3 kb clone (47B). This clone contained a sequence identical to the nucleotide region 38–2920 of the brain CNP-RGC (Chang et al., 1989), a divergent 5′ 37 nucleotide sequence, and an additional 367 nucleotide sequence at the 5′ noncoding region. This DNA was subcloned into the *Eco*R1 site of pBluescript vector in two parts, 47B"A" and 47B"B", which were then ligated to clone 18B resulting in completion of construction of the full-length retinal CNP-RGC (Figure 1).

Clone 18B and subclone 47B"A" were linearized with *Xho*1 and *Not*1, respectively, blunt ended, and cut with *Sph*1. The 1.3 kb fragment of clone 18B was ligated with the 4.15 kb fragment of subclone 47B"A". The ligated plasmid was digested with *Eco*R1 and *Sal*1 and ligated with *Eco*R1–*Sal*1 950 bp fragment of subclone 47B"B" from which the 5′-noncoding region was removed using endonuclease PflM1 and Mung Bean nuclease.

Partial clones corresponding to the human CNP-RGC sequence were isolated also from another human retina cDNA library (λZAP, Stratagene).

GC-B Expression Studies. The expression vector construct (pSVL-CNP-RGC) comprising of the 5′ end of clone 47B and the 3′ end of clone 18B (*Sal*1–*Sac*1-restricted sites) was ligated into the *Xho*1–*Sac*1 site of the pSVL expression vector (Pharmacia).

COS-7 cells (simian virus 40-transformed African green monkey kidney cells), maintained in Dulbecco's modified Eagle's medium with penicillin, streptomycin, and 10% fetal bovine serum, were transfected with the expression vector by the calcium phosphate technique (Sambrook et al., 1989). Sixty hours after transfection, the cells on 100-mm culture plates were washed twice with 50 mM Tris-HCl, pH 7.5, and 10 mM $MgCl_2$, scraped into 2 mL of cold buffer, homogenized, centrifuged for 15 min at 5000*g*, and washed with the same buffer; the pellet represented the crude membranes. These membranes, as indicated, were treated with a series of concentrations of CNP or ANF for 10 min. Cells treated identically, except that they were transfected with the pSVL vector alone, served as control. The guanylate cyclase activity was measured as described earlier (Paul et al., 1987).

RESULTS AND DISCUSSION

Through three consecutive screenings of human retina cDNA library, we characterized two partial clones. The nucleotide sequence of one clone matched the nucleotide sequence of the region 2160-poly(A)$^+$, and that of the other clone matched the sequence 38–2920 of the human brain CNP-RGC (Chang et al., 1989). [The referred human brain CNP-RGC (Chang et al., 1989) was actually cloned from the human glioma cDNA library]. After excising the superfluous overlapping regions, the two clones were ligated to construct the full-length clone, whose coding region sequence was identical to the published brain CNP-RGC sequence (Chang et al., 1989): In both cloned receptor cDNAs, the eukaryotic translation initiation codon (located at the nucleotides 651-653 of human retina CNP-RGC) determines the initiation of a 1047 amino acid open reading frame; the first 22 amino acids represent the N-terminal hydrophobic signal peptide. Excluding the putative signal-peptide sequence, the calculated molecular weight of the protein is 115 331. The sequence topology is consistent with those of the ANF-RGC (Chinkers et al., 1989; Duda et al., 1991) and GCα (Duda et al., 1991), in which the N-terminal 433 amino acids constitute the extracellular domain, followed by a 23 amino acid transmembrane domain that leads into an Arg-Lys stop transfer sequence (Sabatini et al., 1982) and then continues into the 568 amino acid sequence, representing the intracellular portion of the protein. The intracellular part of the protein can be further divided into a kinase-like domain which is followed by a guanylate cyclase catalytic domain. The sequence of the extracellular domain reveals six cysteine residues and seven potential sites for N-linked glycosylation. There is one potential N-linked glycosylation site and nine cysteine residues in the cytoplasmic portion of the protein.

In the encoded region, there are minor structural differences in two of the codons that do not affect the nature of the amino acids: in retina, Leu^{-17} is encoded by CTC and by CTT in brain, and Tyr^{576} is encoded by TAT in retina and by TAC in brain.

There are three major differences between the cDNA sequences of the human retinal and the brain CNP-RGC (Chang et al., 1989) at the noncoding regions: (1) the retinal cDNA contains an additional stretch of 367 nucleotides at the 5′ region; (2) this stretch is followed by a 37-nucleotide sequence that is different from the corresponding brain sequence; and (3) the 3′ coding region in the retinal clone has an additional sequence of 20 nucleotides (Figure 2)

To verify the presence of the divergent sequence in the human genome, primers corresponding to the nucleotides 251–270 and 500–520 of the cloned 5′ noncoding region were used in amplifying the human genomic DNA (Stratagene) by standard PCR reaction. Sequencing of the amplified fragment revealed the presence of the 37 nucleotides divergent sequence noted above (data not shown).

Though human retinal mRNA analysis for the expression of CNP-RGC was not performed, cloning of CNP-RGC from two independent cDNA libraries provides evidence for the expression of CNP-RGC in retina.

Studies with ANF-RGC show that ATP is obligatory in ANF signaling. ANF by itself is unable to stimulate the

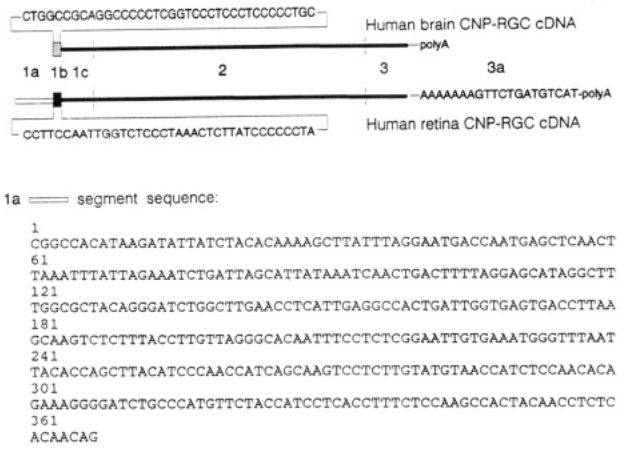

FIGURE 2: Sequences of the new portions of human retina CNP-RGC cDNA. In this diagram the cDNA sequence segments of human retinal and brain CNP-RGC cDNAs are compared. The 1a segment represents a 5′ noncoding 367-nucleotide sequence that is not reported for the brain; the 1b segment represents a 37-nucleotide stretch whose sequence is different from its counterpart in the brain; 1c, 2, and 3 represent, respectively, the 5′ noncoding, coding, and 3′ noncoding regions identical in both the retina and the brain; the 3a segment is the 3′ noncoding 20-nucleotide segment of retinal cDNA which is not reported for the brain cDNA.

membrane guanylate cyclase activity (Chinkers et al., 1991; Marala et al., 1991). Through a program of site-directed and deletion mutagenesis/expression studies, the identity of a structural motif (Gly^{503}-Xaa-Gly-Xaa-Xaa-Xaa-Gly^{509}) that defines an ATP regulatory module of guanylate cyclase has been defined. ATP binds to this sequence and amplifies the ANF-dependent cyclase activity. ATP or ANF, alone, cannot signal an increase in cyclase activity; the presence of both of these agents is necessary (Goraczniak et al., 1992; Marala et al, 1992). To determine the ligand specificity and the role of ATP in CNP signaling, expression vectors containing retinal CNP-RGC (pSVL-CNP-RGC) and ANF-RGC [pSVL-ANF-RGC; see Duda et al. (1991)] were incorporated into COS-7 cells. The particulate fractions of these cells were appropriately treated and analyzed for cyclase activity.

Because earlier studies with ANF-RGC have indicated that maximal guanylate cyclase activity is attained with 0.1 μM ANF in the presence of 800 μM ATP (Marala et al., 1991, 1992), we first used the same concentrations of the above reagents to assess the basal and the CNP-stimulated CNP-RGC activity. The plasma membranes of the CNP-RGC cDNA-transfected cells showed a 50-fold higher basal cyclase activity than the membranes of control cells transfected with pSVL alone, indicating that the encoded protein is a guanylate cyclase (Figure 3). Neither ATP nor CNP altered the basal cyclase activity, but ATP in the presence of CNP increased the cyclase activity in excess of 12-fold. ANF (0.1 μM) in the presence of ATP (800 μM) had no effect on the CNP-RGC cyclase activity, although it caused a more than 12-fold stimulation of ANF-RGC activity; CNP plus ATP had no effect on the ANF-RGC activity. These results indicated specificity and obligatory requirement of ATP in activating CNP-RGC.

To determine the ATP-dependent kinetics of CNP-RGC, the plasma membranes of CNP-RGC-transfected cells were incubated with a series of concentrations of ATPγS, a nonhydrolyzable analog of ATP, in the presence of CNP (0.1 μM). ATPγS stimulated the cyclase activity in a dose-dependent fashion, with the maximal activity expressed at 800 μM and the half-maximal activation (EC_{50}) occurring at $\sim$500 μM ATPγS (Figure 4). Similar results were obtained

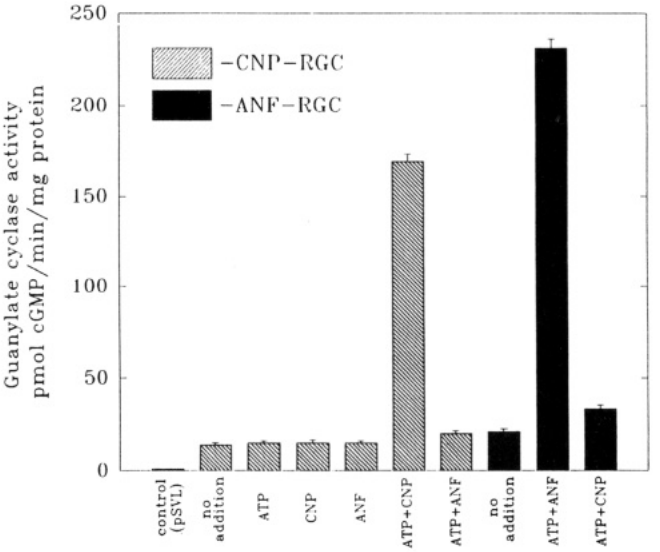

FIGURE 3: Effect of CNP, ANF, and ATP on particulate guanylate cyclase activity in the membranes of COS-7 cells transfected with human retinal CNP-RGC cDNA or genetically constructed ANF-RGC cDNA. Membranes of COS-7 cells transfected with CNP-RGC cDNA (cross-hatched bars) or genetically constructed ANF-RGC cDNA (black bars) were incubated with the indicated additions (CNP or ANF at 10^{-7} M; ATP at 0.8 mM) for 10 min on an ice bath. Guanylate cyclase activity was assayed as described under Experimental Procedures using Mg^{2+} as a cofactor. Experiments were performed in triplicate and repeated three times. Results (mean ± SD) shown are from one typical experiment.

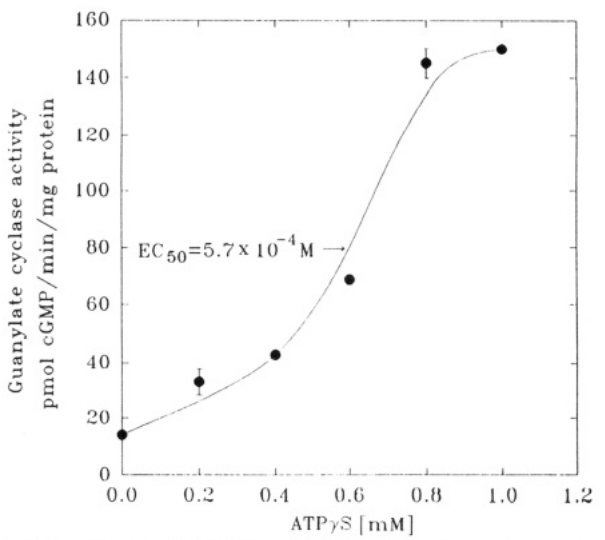

FIGURE 4: Effect of ATPγS on CNP-dependent guanylate cyclase activity in the membranes of COS-7 cells transfected with human retinal CNP-RGC cDNA. Membranes of COS-7 cells transfected with CNP-RGC cDNA were incubated with the indicated concentrations of ATPγS in the presence of 10^{-7} M CNP. Guanylate cyclase activity was measured using Mg^{2+} as a cofactor as described under Experimental Procedures. Experiments were performed in triplicate. Similar results were obtained in another experiment of the same design.

with ATP (data not shown). For comparison, the EC_{50} of ATP for ANF-RGC in the presence of 10^{-6} M ANF was $\sim$300 μM.

To determine the specificity and kinetics of CNP-dependent activation of CNP-RGC, the membranes of the transfected cells were incubated with different concentrations of CNP, or ANF, in the presence of saturating amounts of ATP (800 μM). CNP stimulated the guanylate cyclase activity in a dose-dependent fashion (Figure 5); the EC_{50} concentration was 2 nM, with an excess of 3-fold stimulation occurring well

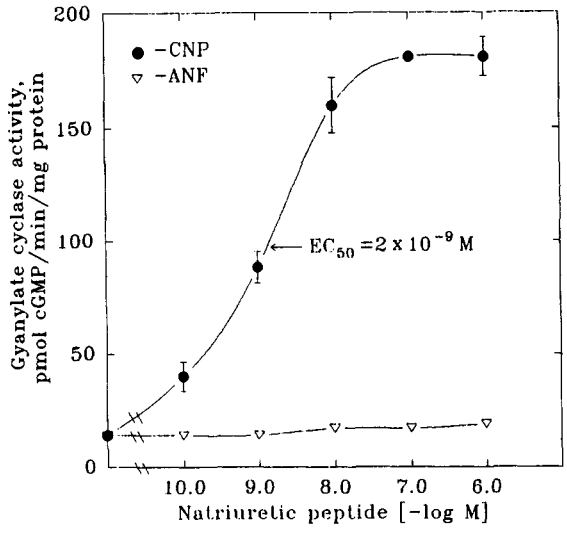

FIGURE 5: CNP- and ANF-dependent guanylate cyclase activity in the membranes of COS-7 cells transfected with human retinal CNP-RGC cDNA. Membranes of COS-7 cells transfected with CNP-RGC cDNA were incubated with various concentrations of CNP or ANF in the presence of 0.8 mM ATP. Guanylate cyclase activity was assayed using Mg^{2+} as substrate. Experiments were done in triplicate and repeated three times. Results (mean ± SD) are from one representative experiment.

below 10^{-10} M. Under identical incubation conditions, ANF had no effect on CNP-RGC activity. For comparison, the EC_{50} concentration of ANF in the ANF-RGC stimulation was about 5 nM.

It is, therefore, established that retinal CNP-RGC meets the pharmacological criteria of being a specific receptor of CNP and that, just like in ANF-RGC signaling, ATP is obligatory in CNP signaling.

These results, in reference to the hormonal sensitivity of human retinal CNP-RGC activity, deserve a comment. To our knowledge, it is the first study conducted directly with the membrane guanylate cyclase fractions. A previous study with the brain receptor was in whole cells; thus the total cyclic GMP formation was assessed in response to the various hormonal stimuli (Koller et al., 1991). In that intact cell study, the potency of CNP in activating the cyclase is 2 orders of magnitude lower than the one observed in the present study; similarly, in the intact cell studies, there was little or no rise in cyclic GMP levels between 10^{-10} and 5×10^{-9} M CNP. In contrast, the retinal guanylate cyclase activity is stimulated almost 3-fold in response to 10^{-10} M CNP in the present study.

The shared feature of an obligatory ATP-mediated event bridging the ligand binding and amplification of cyclase catalytic activities of both ANF-RGC and CNP-RGC suggests two things: (1) that the transduction mechanisms of ANF and CNP receptor-mediated signals are similar and (2) that CNP-RGC, like ANF-RGC (Goraczniak et al., 1992), also contains an ARM where ATP binds and potentiates the CNP-binding signal, which then is transduced at the catalytic site of guanylate cyclase. Structural analysis of the CNP-RGC shows that a Gly^{499}-Xa-Xa-Xa-Gly^{503} sequence in ARM is shared by both CNP-RGC and ANF-RGC. This might, therefore, represent the core ARM sequence of both forms of guanylate cyclases, ANF-RGC and CNP-RGC.

In conclusion, the present study reveals two important points: (1) the human retina contains a CNP signal transduction guanylate cyclase system, suggesting a neuromodulatory role for CNP, and (2) as in ANF signaling, ATP is obligatory in CNP signaling also. The latter observation provides an important clue to the potential identity of the ARM sequence that binds ATP and amplifies the hormonal signal.

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