Identification of a novel aminergic-like G protein-coupled receptor in the cnidarian \textit{Renilla koellikeri}

Christelle Bouchard\textsuperscript{a,b,1}, Paula Ribeiro\textsuperscript{c}, François Dubé\textsuperscript{b}, Christian Demers\textsuperscript{b}, Michel Anctil\textsuperscript{a,}\textsuperscript{*}

\textsuperscript{a}Département de sciences biologiques, Université de Montréal, C.P. 6128, Succ. Centreville, Montréal, Québec, Canada H3C 3J7
\textsuperscript{b}Département d’obstétrique-gynécologie, Université de Montréal and Centre de recherche, Centre hospitalier de l’Université de Montréal (CHUM)-Hôpital Saint-Luc, Montréal, Québec, Canada
\textsuperscript{c}Institute of Parasitology, McGill University, Sainte-Anne-de-Bellevue, Québec, Canada

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Abstract

Biogenic amines exert various physiological effects in cnidarians, but the receptors involved in these responses are not known. We have cloned a novel G protein-coupled receptor cDNA from an anthozoan, the sea pansy \textit{Renilla koellikeri}, that shows homology to mammalian catecholamine receptors and, to a lesser extent, to peptidergic receptors. This putative receptor, named Ren2, has a DRC pattern that replaces the well-conserved DRY motif on the cytoplasmic side of the transmembrane III and lacks the cysteine residues usually found in the second extracellular loop and C-terminus tail. Both the second extracellular loop and the N-terminal tail were seen to be short (six and three amino acids, respectively). Northern blot analysis suggests that the receptor gene codes for two transcripts. Localization of these transcripts by in situ hybridization demonstrated abundant expression in the epithelium of the pharyngeal wall, the oral disk and tentacles as well as in the endodermal epithelium lining the gastrovascular cavities.

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1. Introduction

G protein-coupled receptors (GPCRs) bind ligands present in the extracellular milieu and couple this binding to the activation of intracellular G proteins. These membrane polypeptides are organized into several classes or families according to their sequence homology, identity of crucial residues and motifs that participate in ligand binding, mode of action and pharmacology. The rhodopsin-like family, also named class 1 or family A receptors, contains aminergic and small peptide receptors, and is among the largest families of GPCRs. All members of this GPCR family have a common topology that consists of seven transmembrane (TM) domains separated by three extracellular and three intracellular loops. A number of highly conserved residues, generally found in the TMs, are thought to be required for structural integrity and activation of the receptor within cell membranes.

Neuroactive class I GPCRs, particularly aminergic receptors, are widely distributed across phylogeny. In the ancient metazoan phylum Cnidaria, biogenic amines are considered to be involved in several behaviours, such as feeding (Hanai and Kitajima, 1984), muscular contraction (Anctil, 1989; Anctil et al., 1991; Tsang et al., 1997), metamorphosis (Edwards et al., 1987) and regeneration (Lenicque and Feral, 1994).
One of the best studied effector systems is the control of bioluminescence in the sea pansy *Renilla koellikeri* (Pennatulacea) (Anctil et al., 1982; Awad and Anctil, 1993a). Light emission in this animal is neurally controlled via an adrenergic system. Radiobinding studies have revealed the existence of two high-affinity, adrenergic-sensitive binding sites in membrane homogenates of *Renilla* (Awad and Anctil, 1993b). Whether the receptors are related to the vetebrate β1 and β2 adrenergic subfamilies could not be established unequivocally. Identification of cnidarian receptor subtypes based solely on specific affinity for mammalian receptors is problematic due to the considerable evolutionary divergence between cnidarians and mammals.

In addition to adrenergic-like receptors, putative serotonegic (Hajj-Ali and Anctil, 1997) and dopaminergic receptors were characterized in cnidarians (Hanai and Kitajima, 1984; Chung and Spencer, 1991). Though serotonin (5-HT) enhances the amplitude of rhythmic contraction in the rachis, a disk-shaped colonial mass supporting the polyps of *Renilla* (Anctil, 1989), the action of the serotonergic network on muscular contraction has not been fully elucidated. Other studies, which have characterized dopamine-like receptors in cnidarians, have not explained how the activation of these receptors elicits a response in these animals (Hanai and Kitajima, 1984; Chung and Spencer, 1991).

Despite evidence for the presence of aminergic-like receptors in cnidarians, very little is known about their structure at the molecular level. Recently, we reported the cloning and characterization of Ren1, an amine-like receptor that showed constitutive activity when expressed in mammalian cells (Bouchard et al., 2003). This was the first receptor of its kind to be cloned from a cnidarian, and confirmed that aminergic-like GPCRs appeared early in evolution. Here, we describe a second *Renilla* GPCR-like receptor, named Ren2, which is also homologous to amine receptors and appears to be enriched in tentacles, pharyngeal epidermis, and endodermal epithelium lining the gastrovascular cavities.

## 2. Materials and methods

### 2.1. Polymerase chain reaction (PCR) amplification of a partial Ren2 sequence

Colonies of *Renilla koellikeri* Pfeffer supplied by Marinus (Long Beach, CA) were maintained in artificial sea water, and polyps were collected as described (Bouchard et al., 2003). Genomic DNA was isolated using cetyltrimethylammonium bromide and subjected to a first PCR with degenerate oligonucleotide targets sequence found in transmembranes TMV1 and TMVII of biogenic amine receptors. PCR was performed in a reaction volume of 50 μl containing 20 mM Tris–HCl (pH 8.4), 1.5 mM MgCl2, 2 units of Taq polymerase (Invitrogen, Mississauga, Ontario, Canada) and 200 ng each of primer #1 (sicgityitiitgtgygggyticitcittytt) and #2 (tcigwiwraatiityrataitayigrirtt). The cycling protocol was as follows: initial incubation of 2 min at 94 °C, followed by 40 cycles of 1 min at 94 °C, 1.5 min at 40 °C, 2.5 min at 72 °C, with a final 20-min incubation at 72 °C. The amplification product, a band of 175 bp, was cloned in pCR2 vector (Invitrogen) according to the manufacturer’s protocol. The insert was sequenced and found to be homologous with biogenic amine GPCRs from other species. It was labelled with digoxigenin (DIG) (Roche Diagnostics, Laval, Québec, Canada) and served as a probe to screen for full-length receptor cDNA.

### 2.2. Rapid amplification of cDNA ends (RACE)

Total RNA from *Renilla* polyps was reverse-transcribed with a modified oligo dT primer (gtcaagctagcatagagggctgccagagecctaaagc(T)15) that contained a sequence adapter for use in RACE procedures. The resulting cDNA was subjected to PCR amplification with specific oligonucleotide #3 (taaagcttaaccactaaatcg) and primer RACE-1A (etttagctgctttagc), which hybridized to the adapter sequence (refer to Fig. 1 for the positions of all relevant primer sequences). PCR conditions remained the same except for annealing temperature, which was increased to 50 °C. Southern blot analysis of the PCR products with the DIG-labelled partial Ren2 fragment described above identified two positive bands of approximately 750 and 950 bp. Cloning and sequencing showed that the fragments were identical except for four nucleotides in the non-coding region and an additional 200 bp of predicted untranslated sequence at the 3′ extremity of the longer species. The coding region of the cDNA sequence was homologous with aminergic GPCRs, as determined by a basic local alignment search tool (http://www.ncbi.nlm.nih.gov) with sequence databases at the National Center for Biotechnology. The remaining S′ end of the receptor was obtained by PCR amplification performed on a *Renilla* cDNA library. The library was previously prepared in Lambda ZapII (Stratagene, LaJolla, CA) from oligo dT reverse-transcribed *Renilla* polyps mRNA. An antisense primer #4 (tccttacctcttttgac), deduced from the S′ region of the receptor sequence, and primer T3 (aatataacttctcataagg) that hybridized to the phage arm, allowed the amplification of a 683-bp fragment. Antisense primer #5 (aggacagacatatcataactatatc) employed with primer T3, generated, by PCR, 480 bp of a non-coding sequence and the remaining part of the S′ coding region, as determined by the translational start codon. The S′ end of the coding region was confirmed by cloning and sequencing a second internal fragment obtained by PCR with primers #6 (gaaatctttgacacgac) and T3 (see Fig. 1).

### 2.3. Northern blot analysis

Total RNA was prepared from the polyps as described above, and poly(A)+ RNA was isolated on oligo (dT)-
cellulose (Sigma, Mississauga, Ontario, Canada) according to standard protocols. Aliquots containing 8 μg/lane poly(A)+ RNA were separated on 1% agarose formaldehyde denaturing gel, transferred to a Zeta probe nylon membrane (BioRad, Mississauga, Ontario, Canada) and hybridized with a [32P]-labelled fragment encompassing nucleotides 619 to 729 of Ren2. The membrane was hybridized at 60 °C overnight in 0.25 M Na2HPO4, pH 7.2, 1 mM EDTA and
7% SDS. It was then developed twice at room temperature in 0.1 M Na₂HPO₄, pH 7.2, 1 mM EDTA and 5% SDS, and twice at 60 °C for 20 min in 0.04 M Na₂HPO₄, pH 7.2, 1 mM EDTA and 5% SDS. The blot was exposed to X-ray film for 1 week at ~80 °C with an intensifying screen.

2.4. In situ (IS) reverse transcriptase-polymerase chain reaction (RT-PCR)

Renilla polyps were fixed overnight at 4 °C in 4% paraformaldehyde (PF) in 0.1 M PBS. Eight-micrometer paraffin-embedded sections were mounted onto silicate-coated slides (Labscientific, Livingston, NJ), dewaxed in xylene and dehydrated through a series of ethanol dilutions. The tissue sections were deproteinated with 50 µg/ml proteinase K (Sigma) for 15 min at 37 °C, fixed in 4% PF-PBS for 20 min at 4 °C, then rinsed once in PBS and twice in DEPC-water (diethyl pyrocarbonate) (Sigma). IS RT-PCR was performed using the OneStep RT-PCR kit (QIAGEN, Mississauga, Ontario, Canada) with 0.6 µM of the following primer pairs: forward primer ggggactagtgaattcgcgactgctttca or reverse primer ggggccatgga-caccctgcttctgtgttca. Seventy microliters of reagent mix were added to each slide, and the preparation was covered with a glass cover slip sealed with Aquaperm (Lipshaw Teddington, Middlesex, UK). Reverse transcription was undertaken with NBT-2 emulsion (Kodak, New Heaven, MI). The thermal reaction in a Hybraid thermal cycler (Hybraid, 3. Results

3.1. cDNA cloning

The approach taken to sequence the novel Renilla receptor, Ren2, was based on degenerate PCR amplification that targeted highly conserved regions of amine GPCRs, followed by RACE to obtain the 5’ and 3’ ends of the transcript. The open reading frame of 969 bp of Ren2 encodes a protein of 323 amino acids (aa) with a calculated molecular weight of 36, 364 Da. cDNA extends through the putative ATG initiator, which is flanked by a predicted translation initiation site (Mankad et al., 1998). At the 3’ end, we detected a translation stop codon (TAA) and poly(A)⁺ tail, suggesting that the transcript was full length (Fig. 1).

3.2. Identification of the Ren2 receptor

Hydroporphic domains of the deduced aa sequence of Ren2 were identified by version 2 of the HHMMPH program (Tusnady and Simon, 2001). The positions of the TM boundaries were further adjusted by comparison with the crystal structure of bovine rhodopsin (Palczewski et al., 2000) and an existing model for the mammalian β-adrenergic receptor (Dohlman et al., 1987). This analysis indicated seven hydrophobic domains alternating with more polar regions, characteristic of the seven-membrane spanning topology of GPCRs. The divergent third cytoplasmic loop, which participates in G-protein activation, is only 20 aa long. In contrast, Ren2 possesses a long C-terminus of 65 residues (Karnik et al., 2003).

Consensus N-glycosylation sites occur on the third extracellular loop (N226) and at position N93, in the TMIII domain, although this latter position is unlikely to be glycosylated. Unlike most GPCRs, the short N-terminal extracellular end of Ren2 (3 aa) does not carry a consensus glycosylation site. Putative protein kinase C (T170, S309) and cAMP-dependent protein kinase phosphorylation sites (S313) are found in the third intracellular loop and C-terminal tail. Phosphorylation of GPCRs by these kinases has been implicated in receptor desensitization after prolonged exposure to agonists.

Ren2 exhibited about the same level of sequence homology (approximately 45% similarity) with different types of biogenic amine GPCRs, particularly adrenergic, dopaminergic and some histaminergic receptors. Conserved regions from a selection of receptors are compared in Fig. 2. Some somatostatin receptors, which also belong to class I
Fig. 2. Alignment of the Ren2 receptor amino acid sequence with a selection of homologues. Renilla REN1 (AY057115), β1-adrenergic B1AR (O42574), β2-adrenergic B2AR (Q9T5T3), dopamine-like D5DR (P35346), histamine H2 (NP 037097), octopamine (AAF37686), somatostatin (P35436) and sea anemone ANTHO (AERGPCR) and ACTINTA (JC7270) receptor sequences are identified by accession number and belong to the species Renilla koellikeri, Xenopus laevis, Felis catus, Takifugu rubripes, Rattus norvegicus, Aplysia californica, Homo sapiens, Anthopleura elegantissima and Actinia spp., respectively. Putative transmembrane regions (I–VII) are denoted by lines above the sequences. The amino and carboxy termini and the third intracellular loop were removed. Multiple sequence alignment was generated by using the program ClustalX and displayed by using the editor program Gendoc.

Alignment is represented by dividing amino acids into six similarity groups: group 1, V, L, I and M; group 2, W, F and Y; group 3, E and Q; group 4, K and R; group 5, Q and E; group 6, S and T. Description of similarity: white fonts on black boxes, 100% identity; white fonts on grey boxes, similarity ≥80%; black fonts on grey boxes, similarity <60%.
GPCRs, show sequence identity with Ren2 but the highest homology scores were obtained from comparisons with amnergic receptors. A comparison of Ren2 to the other Renilla receptor Ren1 reveals that identity among the aa sequences is the same as what is seen with the selected amnergic receptors described above. Aside from the other Renilla receptor, Ren1, no close homologue to Ren2 has been identified in non-bilaterians. The two other cloned cnidarian receptors showed similarity mainly to the peptidergic GPCR family (Nothacker and Grimmelikhuijzen, 1993; New et al., 2000) (Fig. 2).

3.3. Functional studies

Ren2 was cloned and expressed in mammalian cells for ligand screening. However, we have not succeeded in finding a ligand with a variety of receptor activity assays designed to detect both cAMP- and Ca2+-mediated signaling, the two major signal transduction pathways for amine GPCRs (see Bouchard et al., 2003 for details of different activity assays). None of the classical biogenic amines, including dopamine, octopamine, tyramine, 5-HT, histamine and adrenergic agents, or somatostatin, could elicit a response in Ren2-transfected HEK293 or LTK-cells (data not shown). Since Ren2 shares the greatest sequence homology with β-adrenergic receptors, we also conducted a radiobinding assay with membranes prepared from LTK-cells transiently expressing the Ren2 receptor. No specific binding was obtained with the tritiated adrenergic ligands CGP12177 and dihydroalprenolol (data not reported).

3.4. Northern blot

Northern hybridization analysis of poly(A)+ RNA from Renilla polyps disclosed two mRNA species of 3.1 and 1.65 kb each (Fig. 3). The 1.65-kb band is in the range of the predicted size of the Ren2 transcript (1.9 kb) whereas the 3.1-kb band is likely to represent a larger isoform of the transcript. Alternative RNA splicing events, which are common among neurotransmitter receptors in other organisms (Grabowski and Black, 2001), may have produced the two Ren2 species in Renilla.

3.5. Localization of the Ren2 transcripts

The tissue distribution of Ren2 was examined by IS RT-PCR, followed by hybridization with an antisense [32P]-labelled Ren2 RNA probe which corresponds to the non-conserved C-terminal intracellular tail of the receptor. IS hybridization performed on sections that were neither reverse transcribed nor PCR amplified showed slightly less labelling than the ones which were submitted to a RT-PCR (results not shown). In both cases, the results clearly show that Ren2 transcripts are highly expressed in the sea pansy. The high density of receptor transcripts that we observed in the tissue of Renilla is not unusual for a GPCR. In the rat, IS

hybridization studies have reported that some discrete regions of the brain abundantly express specific receptor transcripts (O’Dowd et al., 1996; Lee et al., 1999).

The sea pansy is a colonial animal composed of two types of polyps. The autozooids are feeding and reproductive polyps, and the siphonozooids are reduced polyps responsible for water circulation inside the colonial mass (Lyke, 1965). Polyps have a diploblastic histological organization, i.e., they are constituted of two epithelial layers, ectoderm and endoderm, separated by a loosely cellular, gelatinous layer called mesoglea (Lyke, 1965). The hybridization label was particularly strong in the epithelium of the pharyngeal wall and of the oral disk, as well as in both ectodermal and endodermal layers of the tentacles of autozooids (Fig. 4A). Labelling was localized in the basal part of the mucous and ciliated cells constituting the endodermally derived pharynx lining and oral disk (Fig. 4B). The apical parts of these cells, where the mucus compartment and cilia are located, were weakly or not labelled (Fig. 4B).

There was also labelling in the endodermal epithelium lining the eight gastrovascular cavities of the autozooids (Fig. 4C). This lining comprises septa separating the gastrovascular cavities, and endodermal myoepithelia abut against the polyp wall and pharynx (Fig. 4D). The septa are composed of two (longitudinal and radial) muscle sheets separated by a thin mesoglea, whereas the rest of the lining is made up of circular muscle (Lyke, 1965). Labelling was concentrated as a dense band in the basal part of the musculo-epithelial and digestive cells constituting these endodermal epithelia (Fig. 4B). The muscle extension of these cells and the mesoglea in which they are anchored were unlabelled. The cells are involved in the phagocytosis and digestion of food particles, and in muscle contraction...
Labelling was largely absent from the ectoderm of the autozooid body wall. Control sections treated with RNAse presented consistently low levels of labelling in the seven replicate experiments we performed in this study (Fig. 4D).

4. Discussion

4.1. The GPCR-like sequence encoded by Ren2 belongs to the class I family

No definitive statement has yet been made for the existence of aminergic-based neurotransmission in the earliest metazoan animals endowed with neurons. A better understanding of the aminergic systems of cnidarians requires characterization of their receptors. Physiological and biochemical approaches have so far been the main way of studying receptors for biogenic amines in cnidarians. Identification of the receptor sequences found in these animals and eventually their pharmacological characterizations will lead to possibilities of new research on primitive nervous systems.

We describe here a novel cDNA from *Renilla koellikeri* that encodes a putative amine receptor and the localization of its transcripts in this animal’s polyps. The new receptor, named Ren2, has typical seven TM domain topology, and displays several structural characteristics of class I GPCRs. In particular, the motifs F<sup>203</sup>xxxWxPFF in TMVI (Shi and Javitch, 2002) and N<sup>246</sup>SxxNPxxY (Oliveira et al., 1999) of TMVII are signatures of receptors of the rhodopsin-like family. There are, however, several noteworthy differences between Ren2 and other members of the family. Instead of the highly conserved DRY motif found at the C-terminal region of TMIII, Ren2 displays a D<sup>100</sup>RRC pattern at this position. A cysteine substitution at the third position of this motif is known to occur in only 4% of class I GPCRs (Van Rhee and Jacobson, 1996). A second interesting feature of Ren2 is the absence of highly conserved disulfide linkage between extracellular loops (EL) 1 and 2. Only one of the two conserved cysteines involved in this linkage is present in Ren2 (C<sup>78</sup>). The absence of the disulfide bond may be
related to the exceptionally short length of EL2 (6 aa) in Ren2. It will be of interest to determine if/how these fundamental structural differences affect the activity and stability of the receptor in vivo.

Ren2 also exhibits many of the conserved residues believed to play a role in GPCR binding and activation. Notably, aspartic acid D50 in TMII may regulate agonist binding affinity (Strader et al., 1988; Van Rhee and Jacobson, 1996) and, together with asparagine N22, may determine if any of the different ligand preferences. It will be of interest to identify any of the “non-standard” amine substances, previously identified in cnidarians (Elofsson et al., 1977; Carlberg and Rosengren, 1985), are capable of activating Ren2.

4.3. Ren2 transcripts are abundant in polyps

Feeding in the sea pansy is accomplished by capturing small prey with its tentacles and by their ingestion through the mouth. The food particles are moved in the gastrovascular cavities until they are captured by the digestive cell bodies of the endodermal myoepithelium (Lyke, 1965). The predominance of Ren2 labelling in key tissues supporting these activities (tentacles, oral disk, pharynx cells, and digestive cells of myoepithelia) suggests that the receptor may play a role in feeding, especially in food transport and intracellular digestion. Although Ren2 labelling in endodermal myoepithelia would suggest its involvement in muscle activity, the absence of labelling in the muscle part of the epithelia makes this possibility doubtful and myoepithelial cells display other activities such as phagocytosis for intracellular digestion of food particles (Lyke, 1965). Few studies have documented the effects of biogenic amines on feeding. Physiological studies on Hydra and C. elegans have discovered modification of the feeding response upon exposure to biogenic amines (Hanai and Kitajima, 1984; Nuttley et al., 2002). However, pending identification of a ligand for the Ren2 receptor, it is difficult to speculate further about its role in this animal.

4.4. Evolutionary implications

From an evolutionary standpoint, cnidarians represent an ancient metazoan group. Given the basal position that the anthozoan class occupies in the phylum (Bridge et al., 1992), Ren1 and Ren2 can be considered as representatives of the oldest sequences, with the distinct structural characteristics of biogenic amine receptors. The great diversity of 5-HT receptor subtypes in the animal kingdom led Peroutka (1994) to propose that biogenic amine receptors might be the descendants of a primordial 5-HT receptor. However, the Renilla Ren2 receptor, and the recently cloned Ren1 receptor, appear to be more similar to catecholamine receptors than to 5-HT receptors. Discoveries of more GPCR sequences from primitive phyla and identification of their ligands might shed light on the origin of amine GPCRs in eukaryotes.

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