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Biochemical and Biophysical Research Communications 344 (2006) 160–165
BBRC
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Identification of four evolutionarily related G protein-coupled receptors from the malaria mosquito *Anopheles gambiae* ☆

Martin Belmont, Giuseppe Cazzamali, Michael Williamson, Frank Hauser, Cornelis J.P. Grimmelikhuijzen *

Center for Functional and Comparative Insect Genomics, Department of Cell Biology and Comparative Zoology, Institute of Biology, University of Copenhagen, DK-2100 Copenhagen, Denmark

Received 16 March 2006

Abstract

The mosquito *Anopheles gambiae* is an important vector for malaria, which is one of the most serious human parasitic diseases in the world, causing up to 2.7 million deaths yearly. To contribute to our understanding of *A. gambiae* and to the transmission of malaria, we have now cloned four evolutionarily related G protein-coupled receptors (GPCRs) from this mosquito and expressed them in Chinese hamster ovary cells. After screening of a library of thirty-three insect or other invertebrate neuropeptides and eight biogenic amines, we could identify (de-orphanize) three of these GPCRs as: an adipokinetic hormone (AKH) receptor (EC_{50} for *A. gambiae* AKH, 3×10^{-9} M), a corazonin receptor (EC_{50} for *A. gambiae* corazonin, 4×10^{-9} M), and a crustacean cardioactive peptide (CCAP) receptor (EC_{50} for *A. gambiae* CCAP, 1×10^{-9} M). The fourth GPCR remained an orphan, although its close evolutionary relationship to the *A. gambiae* and other insect AKH receptors suggested that it is a receptor for an AKH-like peptide. This is the first published report on evolutionarily related AKH, corazonin, and CCAP receptors in mosquitoes.

Keywords: GPCR; DRY motif; Neuropeptide; Neurohormone; GnRH; Endocrinology; Malaria; Insect; *Drosophila*; Mosquito; Silkworm

Malaria is one of the most serious parasitic diseases in the world, responsible for up to 500 million cases of illness and 2.7 million deaths (mostly children) each year. The disease is caused by an infection with the intracellular parasite *Plasmodium falciparum*, which is transmitted by the malaria mosquito, *Anopheles gambiae* [1]. To find new strategies for malaria control, both the genomes from *P. falciparum* and that from *A. gambiae* were sequenced recently [2,3]. Exploiting these genomic data will open up new possibilities to fight *P. falciparum* or its vector *A. gambiae* with high selectivity and low human toxicity.

G protein-coupled receptors (GPCRs) are well-known drug targets. More than 30% of all prescribed human medication is acting on this large family of membrane proteins [4]. GPCRs are structurally characterized by the presence of seven transmembrane α-helices, and they have a key function in cell communication by linking extracellular signalling substances (hormones or neurotransmitters) to intracellular effector proteins. In all animals, GPCRs and their ligands steer important physiological processes such as development, reproduction, feeding, and behavior.

By analysis of the sequenced *Anopheles* genome, 276 genes could be annotated to code for GPCRs. Twenty-five of these GPCRs were annotated as putative neuropeptide receptors belonging to the family of rhodopsin-like or class A GCPRs [5,6]. Until today, nearly all of these neuropeptide receptors are orphans, because their ligands are

☆ The sequence data of this paper have been submitted to the GenBank database under Accession Nos. AY298745, AY301275, AY500851, and AY553322.

* Corresponding author. Fax: +45 3532 1200.
E-mail address: cgrimmelikhuijzen@bi.ku.dk (C.J.P. Grimmelikhuijzen).
URL: http://www.bi.ku.dk/staff/staff-vip-details.asp?ID=90 (C.J.P. Grimmelikhuijzen).

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doi:10.1016/j.bbrc.2006.03.117

unknown. To understand their biological functions, they have to be deorphanized (the ligand identified), using the orphan or reverse pharmacology strategies [7,8]. Likely ligand candidates for the twenty-five neuropeptide receptors can be found within the thirty-five annotated *Anopheles* genes, encoding putative peptide preprohormones [9].

In this paper, we describe the molecular cloning and expression in Chinese hamster ovary (CHO) cells of four evolutionarily related mosquito GPCRs. For three of them we could identify the endogenous ligands (namely, adipokinetic hormone (AKH), an insect neuropeptide involved in carbohydrate and lipid mobilization during flight; corazonin, a neuropeptide involved in heart beat; and crustacean cardioactive neuropeptide (CCAP), a neuropeptide involved in heart beat and molting), whereas one receptor remained orphan.

Materials and methods

Total RNA was extracted from adult *A. gambiae*, which were grown in our laboratory (strain KWA, kindly supplied by Drs. N. Hill and P. Aiyenuro, London School of Hygiene and Tropical Medicine, UK), using Trizol reagent (Invitrogen). cDNA was made, using the SMART RACE cDNA amplification kit (Clontech). This kit was also used for the RACE reactions. All other PCRs were carried out, using the Herculase enzyme system (Stratagene). The following primers were applied: AKH receptor, 5′-AAAATGCCCAACACAATGGCCGCCCACATCAAC and 5′-CTA CGCCCTCATCGTCAGATGACTGCCGCAGCT; corazonin receptor, 5′-GCGGCAAAATGCCGCCATCGTTCAACCTCTCC and 5′-CCGG TTTAAAGCTTAGTCGGATGGTTTCGTTGC; CCAP receptor, 5′-GT GCGCGCGGAACCATGCTGCCAATTCTTACC and 5′-GCGCACCC TTCCCTCTACACCTCCGACATGGC; and orphan receptor, 5′-GGAG GCAGCCAACCAGTCGGGATGTACCTG and 5′-CCGTCCAGTAG AAAGCAAGCACACTACC. The PCR products were ligated into the pCR4–TOPO vector (Invitrogen) and sequenced. The coding regions were subcloned into the expression vector pIRES2–EGFP (Clontech) using *Eco*RI restriction sites.

The resulting constructs were transfected into Chinese hamster ovary cells, stably expressing the promiscuous human G protein $G_{\alpha-16}$ (CHO-G16) using FuGENE6 transfection reagent (Roche). The transfected cells were cloned and those with the highest fluorescence were chosen for further studies. Two days prior to the assay, cells were transiently transfected with DNA coding for apoaequorin. Three hours prior to the assay, 5 μM coelenterazine was added to the culture medium. Activation of an expressed receptor in these pretreated cells would initiate an IP_3/Ca^{2+} cascade, leading to a strong bioluminescence response [10–13].

The tested compound library consisted of 26 *Drosophila* neuropeptides (AKH, allatostatin-A4, -B1, -B2, -B5, and -C, capa-1, -2, and -3, CCAP, corazonin, ecdysis triggering hormone-1 and -2, hug-γ, IPNamide, MTY-amide, myosuppressin, neuropeptide F, pigment dispersing hormone, proctolin, pyrokinin-2, short neuropeptide F1, SIFamide, sulfakinin-0 and -2, and tachykinin-3), seven other invertebrate peptides (allatotropin, *Anopheles* adipokinetic hormone, FMRFamide, hypertrehalosaemic neuropeptide, leucokinin-3, leucopyrokinin, and perisulfakinin) and eight biogenic amines (adrenaline, dopamine, histamine, melatonin, noradrenaline, serotonin, octopamine, and tyramine) dissolved in phosphate-buffered saline (for peptide structures, see [14–16]).

DNA sequence compilation and phylogenetic tree analyses were done using the Lasergene DNA software package (DNASTAR). Protein sequence alignments were carried out using CLUSTALW (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_clustalw.html). EC_{50} values were calculated using Prism software.

Results

By BLAST searching of the *Anopheles* genome sequence [3] with the DNA sequences from the *Drosophila* AKH, corazonin, and CCAP receptors [11,17,18], we identified several DNA fragments, coding for putative *Anopheles* orthologues. Based on this information, we designed primers and cloned the entire cDNAs coding for four *Anopheles* GPCRs, using PCR and RACE-PCR (Figs. S1–S4, presented as supporting material to this paper; and GenBank Accession Nos. AY298745, AY301275, AY500851, and AY553322). All four cDNAs contained a polyadenylation signal in their 3′ untranslated region followed by a poly(A)$^+$ tail. In addition, all cDNAs contained at least one in-frame stop codon preceding the ATG start codon in their 5′ untranslated region. Compared with the genomic DNA sequence, our cDNA sequences showed several nucleotide differences. Some of these differences led to amino acid residue changes in the encoded proteins (Tables S1–S4, supporting material). These comparisons also revealed that the four receptor genes contained several introns: six introns were present in the *Anopheles* AKH-like receptor (Ang-AKHR) gene, five in the corazonin-like receptor (Ang-CORR) gene, eight introns in the CCAP-like receptor (Ang-CCAPR) gene, and six introns in the fourth receptor-like (Ang-Orphan) gene (Tables S5–S8, supporting material).

The amino acid sequences of the proteins encoded by the four cDNAs are shown and aligned in Fig. 1. They all show the typical hallmarks of GPCRs, such as seven transmembrane α-helices and highly conserved sequence motifs (Figs. S1–S4, supporting material). Two GPCRs have the well-known DRY consensus sequence in the intracellular loop directly following TMIII (Ang-AKHR and Ang-CCAPR), whereas Ang-CORR has the conserved DRY variant, DRW. However, Ang-Orphan has a DRC sequence at this position. This variant of the DRY motif is quite unusual. Interestingly, the DRC motif is also present in the AKH receptor from the cockroach *Periplaneta americana* [19].

To deorphanize the four GPCRs, we expressed them individually in CHO-G16 cells. A library of 33 invertebrate neuropeptides and eight biogenic amines was tested on the recombinant stable cell lines, using our bioluminescence assay (see Materials and methods). Ang-AKHR was only activated by various insect AKHs. The most potent peptide was the *Anopheles* AKH (pQLTFTPAWamide) with an EC_{50} of 3×10^{-9} M (Fig. 2). The preprohormone gene, containing this peptide, was identified by a BLAST search of the *Anopheles* genome sequence with AKH sequences from other insects.

Ang-CORR was only activated by *Drosophila* corazonin (pQTFQYSRGWTNamide) with an EC_{50} of 4×10^{-9} M (Fig. 3) and not by other compounds from our library. A gene encoding a peptide identical to *Drosophila* corazonin was previously identified in *Anopheles* [9].

Ang-CCAPR was selectively activated by CCAP (PFCNAFTGCamide) with an EC_{50} of 1×10^{-9} M

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Ang-CORR   ------------------------------------------------MPPSFNLSTVTYELISGNVS     20
Ang-CCAPR  MLPILTWAYLRHIAVQYHAEAQDPYGSVQLVSANPPLGTEHAGHAGYDCASLPLFALPRDIDSSDSGSSH     70

Ang-CORR   AALENFTASMAGAFPFEDLHDPASSIARNASFTLGLGLANVIQLQQQQQQQQLHHSPHQYHQQVQHQPQP     90
Ang-CCAPR  LERTIELTFSPADCRLPPRWTIRNDTFRPFQQSAKLAGGGGLNMRFRDQTDTYTGSAGSSTQIAAFPLDH    140

Ang-AKHR   ------------------------------------MPNTMAAHINQRIEDHRNLADWSYYANETAG     31
Ang-Orphan ----------MYLAAGLLNIMDISLQHEYLQEYLQSAAAMANFSGANPYGLGGFGGLAPNGTGLLGGLDK     60
Ang-CORR   PSTPFANVSTGQNESLANLLLHPGVHQLSSGLVTLVGDIMAQSGDTILPREECDRLNISYAFENGTALEI    160
Ang-CCAPR  SSAAIGESADAAHGSSVAGGLGSVGSFVAVNDVIGMNITPALSQVLLAPPATSAGSGASIIAAVNGAAAD    210
                                    TMI
Ang-AKHR   EEYYEMPIDMRFNSGHILSTMVYTTLMVFSATGNLTVLSILAQRKVRA------SSRINIMLAHLAIADL     95
Ang-Orphan NGTEVTITAPGHTDSTVAVIIVYCVLFVIAAGGNLSVVITLFRSRRHR------RSRVSLMICHLAVADL    124
Ang-CORR   PGLSCYEHAPTLSKSGVIRVIVLSAMAIVSLLGNVATMWNIQKNRKSRRVTRHNWSAIYSLIFHLSIADV    230
Ang-CCAPR  DNGTRGEEINSFYFYETEQFAFLWVLFTVIVLGNSAVLVTLMLNRTR-------KSRMNFFIKQLAIADL    273
           TMII                    TMIII                                        
Ang-AKHR   LVTFLMMPLEIGWAYTVRWTAGDLMCRVMAFFRTFGLYLSSFILICISVDRYFAVLKPLKVHEHR--AVL    163
Ang-Orphan MVAFIMIPLEVGWRITVQWHAGNVACKVFLFMRAFCLYLSSNVLVCVSLDRCFAVIYPLRVSAARKRGKI    194
Ang-CORR   LVTGFCLIGEAAWYYTVDWVAGNLFCKLFKLCQMFSLYLSTYVLVLVGVDRWVAVKYPMKSLNTARRCHR    300
Ang-CCAPR  CVGLLNVLTDIIWRITVVWRAGNAACKAIRFVQVCVTVASTYVLVALSIDRYDAITHPMNFSGCWSRARK    343
           TMIV                                                    TMV           
Ang-AKHR   MIAAAWIMSGLCSLPQAFIFHLEGHPNITGYQQCVTYHYFEEEIYQIIYNVLVMCLMYTFPLIVILYCYG    233
Ang-Orphan MLGGAWFIAFVNAMPQSIIFRVQQHPQVPGFTQCVTFGFFATPGLETAYNLFCVIAMYFLPLMIISGAYT    264
Ang-CORR   FLFVAYLLSFLLSTPQWMIFRVAKGPFVEDFYQCVTHGFYTDRWQEQLYTTFTLVFMFIIPLLILIGTYL    370
Ang-CCAPR  LVAAAWSFSILFSLPITYFYEER---LIQGKMQCWID--LVEAWRWQLYMCWVSGSLFVVPALIISACYA    408
                                                                TMVI               
Ang-AKHR   SIYYEIFSR----------TNPRNLESFRRSSIDVLGRAKRKTLRMTIMIVIVFVVCWTPYYVMSLWYW    292
Ang-Orphan VILCEISNRSREK---ETSDSNSTGTMRLRCNDLTHIERARQRTLRLTITIVVVFVWCWTPYYVMTLWYM    331
Ang-CORR   STFMTISSSEKIFRIDTSAVDRTIYYRRSDTNRQRLIHKAKMKSLRISVVIVVAFVVCWTPYYIMMLIFM    440
Ang-CCAPR  VIVRTIWAKGTILGPIDRTHNGMADLATRRASSRGIIPRAKVKTVKMTIVIVIVFVLCWSPYIIFDLLQV    478
                        TMVII                                                      
Ang-AKHR   LDKESAKNVDQRIQKGLFLFASTNSCMNPVVYGVFNVRKKHTKKLLKTTHEKSCGSHLTMRA--------    354
Ang-Orphan FDRESAAKVDVAVQDGLFLMAVSNSCMNPLVYGSYAMKCRLPCRRRNTLGGAQTPNAAQRRSTGFRCQQR    401
Ang-CORR   FLN-PTERFGEDLQSGIFFFGMSNSLINPLIYGAFHLVPIRQRRNQYNQHVREGSVYFQRSSTFNHHNGH    509
Ang-CCAPR  FGQIPATQTNVAIATFIQSLAPLNSAANPLIYCLFSTQVCRMIKRLPPFRWLWSTKWCCKPHDSGTLPLG    548

Ang-Orphan WDPKMNLRFCMSILKNISFSLLLLW-------------------------------------------    426
Ang-CORR   QRNLNPHIKFSNSHSNLPEEISLMSLEKDLRSLDENVNQIHQKGGGGGGGGGGGGGGVGGGIGLSLGGAA    579
Ang-CCAPR  NGTTVNGTAHGRFHNHNSSDSMRTLTTSLTVSRRSCLRPARVVIVERPKTEHAMSEV-------------    605

Ang-CORR   GVDGSRRIKRRSFASKFLSFSRLLQRNHPTKL                                        611
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Fig. 1. Protein sequence alignment of the adipokinetic hormone-like receptor from *A. gambiae* (Ang-AKHR, GenBank database Accession No. AY298745), the orphan receptor (Ang-Orphan, Accession No. AY553322), the corazonin-like receptor (Ang-CORR, Accession No. AY301275), and the crustacean cardioactive peptide-like receptor (Ang-CCAPR, Accession No. AY500851). Spaces are introduced to optimize alignments. The seven transmembrane α-helices are indicated by TMI–TMVII. Amino acid residues in TMI–TMVII that are identical between at least two of the receptors are highlighted in grey. Intron positions are indicated by boxes.

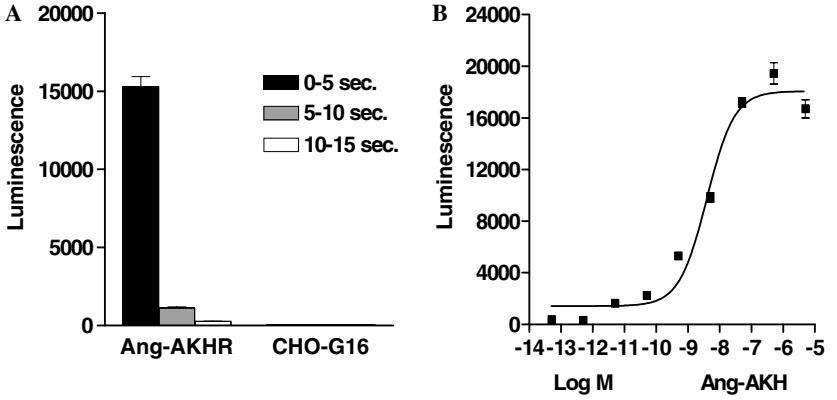

Fig. 2. Bioluminescence responses of recombinant cell lines to *Anopheles* adipokinetic hormone (Ang-AKH). The vertical bars represent SEMs ($n = 4$ in (A); $n = 4$ in (B)), which sometimes are smaller than the symbols (or lines) used. In these cases, only the symbols (or lines) are given. (A) Left panel. Bioluminescence response of a cloned CHO-G16 cell line, stably expressing Ang-AKHR (Ang-AKHR) to 5×10^{-6} M Ang-AKH. Right panel. The absence of a response to 5×10^{-6} M Ang-AKH in non-transfected CHO-G16 cells (CHO-G16). (B) Dose–response curve of the Ang-AKH response in Ang-AKHR cells.

(Fig. 4) and not by any other compounds from our library. A precursor gene, encoding the highly conserved peptide CCAP, was earlier identified in *Anopheles* [9].

The fourth receptor, Ang-Orphan, could not be activated by any compound from our library at concentrations up to 10^{-5} M.

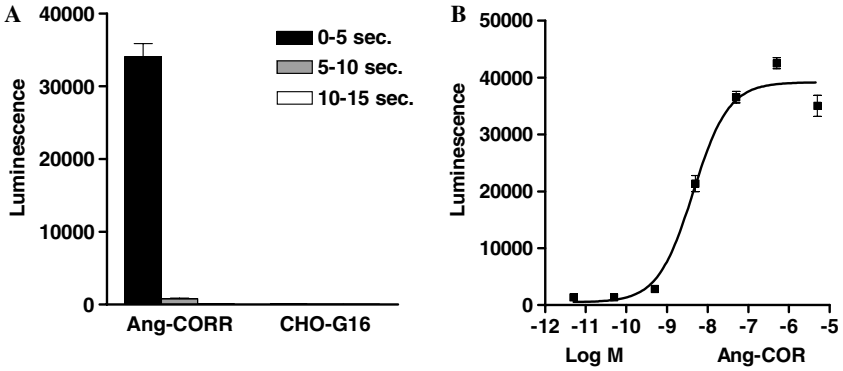

Fig. 3. Bioluminescence responses of recombinant cell lines to *Anopheles* corazonin (Ang-COR). SEMs are presented as in Fig. 2. (A) Left panel. Bioluminescence response of a cloned CHO-G16 cell line, stably expressing Ang-CORR to 5×10^{-6} M Ang-COR. Right panel. The bioluminescence response of non-transfected CHO-G16 cells to 5×10^{-6} M Ang-COR. (B) Dose–response curve of the effects seen in Ang-CORR cells.

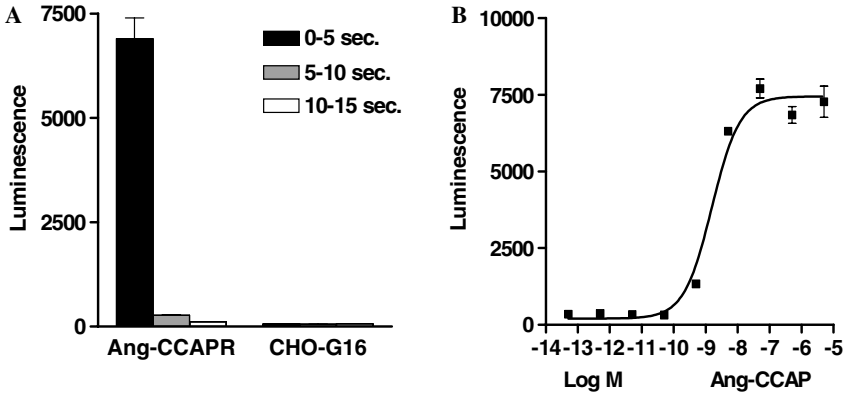

Fig. 4. Bioluminescence responses of recombinant cell lines to *Anopheles* crustacean cardioactive peptide (Ang-CCAP). SEMs are presented as in Fig. 2. (A) Left panel. Bioluminescence response of a cloned CHO-G16 cell line, stably expressing Ang-CCAPR (Ang-CCAPR) to 5×10^{-6} M Ang-CCAP. Right panel. The response of non-transfected CHO-G16 cells to 5×10^{-6} M Ang-CCAP. (B) Dose–response curve of the effects seen in Ang-CCAPR cells.

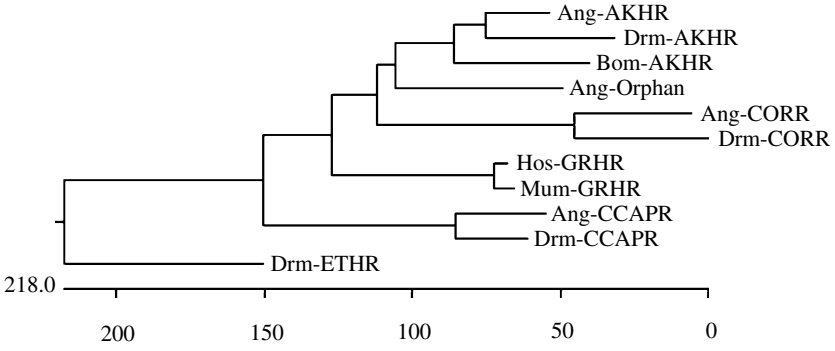

Fig. 5. Phylogenetic tree analysis of the four GPCRs from Fig. 1 and other related receptors. The tree is made using the nearest neighbour-joining method of the DNASTAR megalign software. The length of each branch represents the distance between each receptor and the common ancestor of that receptor and its neighbour. The units at the bottom indicate the number of the amino acid residue substitutions, corresponding to this distance. The eclosion-triggering-hormone receptor from *Drosophila melanogaster* (Drm-ETHR) [21] is used as an outgroup to root the tree. Other abbreviations used (from top to bottom): Ang-AKHR, *A. gambiae* AKH receptor; Drm-AKHR, *D. melanogaster* AKH receptor; Bom-AKHR, *Bombyx mori* AKH receptor; Ang-Orphan, *A. gambiae* orphan receptor; Ang-CORR, *A. gambiae* corazonin receptor; Drm-CORR, *D. melanogaster* corazonin receptor; Hos-GRHR, *Homo sapiens* GnRH receptor; Mum-GRHR, *Mus musculus* GnRH receptor; Ang-CCAPR, *A. gambiae* CCAP receptor; Drm-CCAPR, *D. melanogaster* CCAP receptor.

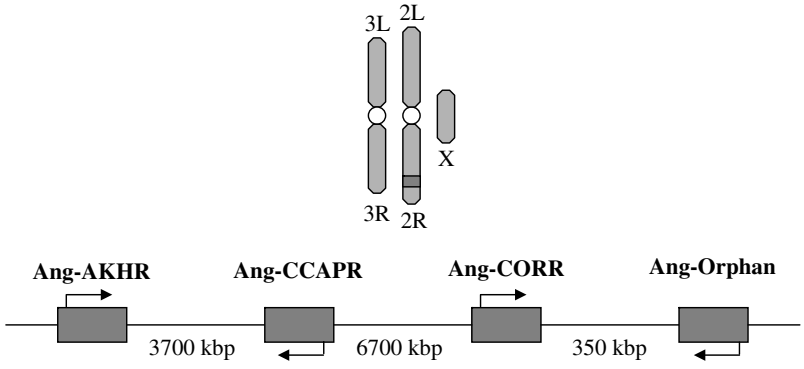

Fig. 6. Schematic drawing of the chromosomal location of the four GPCR genes from Figs. S1–S4. These genes are clustered on the right arm of chromosome 2 and are transcribed in different directions (arrows). The distances are given in kilobasepairs (kbp).

Fig. 1 shows that the four GPCRs are evolutionarily related, because they have a considerable percentage of amino acid sequence identities and similarities (e.g., between Ang-AKHR and Ang-Orphan, 44% identities and 65% similarities in the seven-transmembrane region; Ang-AKHR/Ang-CORR, 29% and 55%, respectively; Ang-AKHR/Ang-CCAPR, 28% and 47%, respectively). In addition, three of the four receptor genes share introns (Fig. 1). The evolutionary relationships between the four GPCRs and similar GPCRs from other species were further analysed, using a phylogenetic tree analysis (Fig. 5). Ang-Orphan showed highest homology to the cluster of AKH receptors from *Anopheles* (Ang-AKHR), *Drosophila* (Drm-AKHR), and *Bombyx* (Bom-AKHR). Ang-CORR is clearly the orthologue of the *Drosophila* corazonin receptor (Drm-CORR) and Ang-CCAPR is the orthologue of the *Drosophila* CCAP receptor (Drm-CCAPR). Insect corazonin and AKH receptors show higher homologies to each other than to the CCAP receptors, which form a separate branch. We have previously noticed that the insect AKH receptors are evolutionarily related to mammalian gonadotropin-releasing-hormone (GnRH) receptors [11,20]. This finding is confirmed in the present analysis, where human (Hos-GRHR) and mouse (Mum-GRHR) GnRH receptors are clearly evolutionarily related to insect AKH receptors, but also to corazonin and CCAP receptors (Fig. 5).

All four *Anopheles* receptor genes are localized in a defined region of chromosome 2R spanning over a distance of about 11 Mbp, supporting the common evolutionary origin of the four genes (Fig. 6).

Discussion

In our present paper, we have cloned four novel GPCRs from the malaria mosquito *A. gambiae*. Three of these GPCRs were deorphanized and identified, respectively, as an AKH, corazonin, and CCAP receptor (Figs. 2–4). The fourth receptor (Ang-Orphan) remained an orphan. However, because of its close phylogenetic relationship to the insect AKH receptors (Fig. 5), it is likely that also this receptor is an AKH receptor. The reason that Ang-Orphan was not activated by insect AKHs might be that it does not interact with the promiscuous G protein, G-16, and that it, therefore, cannot be monitored in our assay system.

The existence of Ang-Orphan in *A. gambiae* is highly interesting, because it shows for the first time that there might be a second AKH receptor in an insect. The existence of more than one type of AKH receptor in an insect is also suggested by the presence of more than one AKH peptide. In cockroaches and locusts, for example, there are two, respectively, three different AKHs [22]. It would, therefore, be worthwhile to investigate, whether also *A. gambiae* has more than one AKH (or AKH-like) peptide.

For *Drosophila melanogaster* it has been noticed that its AKH, corazonin, and CCAP receptors are evolutionarily closely related [17,18,23]. This is interesting, because also the AKH and corazonin peptides are closely related, suggesting that there has been a co-evolution between receptors and their ligands [17,18,23]. Because Ang-Orphan is phylogenetically situated between the insect AKH and corazonin receptors (Fig. 5), it probably also shares the same evolutionary properties, i.e., that the three receptors and their ligands have co-evolved. This implicates that the ligand of Ang-Orphan is likely to be an AKH (or corazonin)-like peptide.

Anopheles gambiae is an important vector of malaria. A selective reduction (or eradication) of the population of this mosquito, therefore, will strongly reduce (or even stop) the transmission of malaria. It is crucial for the selective reduction of *A. gambiae* mosquitoes to understand their physiology and identify appropriate drug targets. GPCRs occupy a central position in the physiology of insects and are excellent drug targets in human medicine [4]. The cloning and characterization of GPCRs in *A. gambiae*, therefore, might pave the way for a selective and environmentally safe control of malaria mosquitoes.

Acknowledgments

We thank Sarah Preisler for typing the manuscript, and the Danish Research Agency (Research Council for Nature and Universe), Carlsberg Foundation, Novo Nordisk Foundation, and Industrialist Vilhelm Pedersen and His Wife's Memorial Legacy for financial support.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2006.03.117.

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