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Co-evolution of Ligand-Receptor Pairs in the Vasopressin/Oxytocin Superfamily of Bioactive Peptides*

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In order to understand the molecular mechanisms that underlie the co-evolution of related yet functionally distinct peptide-receptor pairs, we study receptors for the vasopressin-related peptide Lys-conopressin in the mollusc *Lymnaea stagnalis*. In addition to a previously cloned Lys-conopressin receptor (LSCPR1), we have now identified a novel Lys-conopressin receptor subtype, named LSCPR2. The two receptors have a differential distribution in the reproductive organs and the brain, which suggests that they are involved in the control of distinct aspects of reproduction and mediate transmitter-like and/or modulatory effects of Lys-conopressin on different types of central neurons. In contrast to LSCPR1, LSCPR2 is maximally activated by both Lys-conopressin and Ile-conopressin, an oxytocin-like synthetic analog of Lys-conopressin. Together with a study of the phylogenetic relationships of Lys-conopressin receptors and their vertebrate counterparts, these data suggest that LSCPR2 represents an ancestral receptor to the vasopressin/oxytocin receptor family in the vertebrates. Based on our findings, we provide a theory of the molecular co-evolution of the functionally distinct ligand-receptor pairs of the vasopressin/oxytocin superfamily of bioactive peptides.

Peptide receptors form an important subclass of the otherwise diverse superfamily of G protein-coupled[1] receptors that all have a seven-transmembrane segment topology in common (for review, see Ref. 1). This receptor type forms a component of a modular system for the transduction of extracellular signals across the cell membrane and the subsequent conversion of these signals to an intracellular second messenger pathway via the activation of heterotrimeric G proteins (for review, see Ref. 2). Molecular evolutionary mechanisms such as gene duplication and subsequent mutation of the resulting genes have resulted in the formation of families of related yet distinct peptides (3) and peptide receptors (4). In the nervous and endocrine systems, many peptide isoforms bind to distinct receptor subtypes that mediate the specific cellular actions that underlie a large variety of behavioral and physiological processes. Peptide-receptor and receptor-effector interactions are of critical importance for peptide function, and many diseases are linked to malfunctions of these interactions (5). However, the great variety in structure of both peptides and receptors has hampered the development of a coherent theory explaining the molecular basis of co-evolution of specifically interacting peptide-receptor pairs.

The vasopressin/oxytocin superfamily of peptides and their cognate receptors offer an attractive model for the study of specificity of peptide-receptor interactions and the co-evolution of related peptide-receptor pairs (6). These peptides and receptors occur throughout the entire animal kingdom (for review, see Ref. 7), allowing detailed comparison of their structural features and experimental testing of putative specificity determinants. The peptides of the superfamily are surprisingly alike, sharing at least 5 out of 9 residues and a disulfide-linked ring structure that put severe constraints on conformational flexibility (8, 9). In the vertebrates, gene duplication gave rise to related yet distinct vasopressin and oxytocin genes (10). The different functions of vasopressin and oxytocin are mediated by genetically distinct receptor subtypes (11, 12). The V2 vasopressin receptor mediates the antidiuretic effects of vasopressin and couples positively to adenylate cyclase (13–16). By contrast, the V1a (17, 18) and the V1b (19) vasopressin receptors, which mediate the effects of vasopressin on liver glycogenolysis and on adrenocorticotropin release, respectively, as well as the oxytocin receptor (15, 20, 21), which mediates the various central and peripheral functions in reproduction of oxytocin, all couple to the inositol trisphosphate/Ca^{2+} signal transduction pathway. The discriminative binding of vasopressin and oxytocin to their respective receptors is dictated to a large extent by the amino acid residue at position 8. At this position, the family of vasopressin and related peptides have a basic residue, whereas oxytocin and related peptides have a neutral, in most cases aliphatic residue (7).

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) U40491.

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[1] The abbreviations used are: G protein, guanosine nucleotide binding protein; LSCPR, *L. stagnalis* conopressin receptor; TM, transmembrane domain; RT, reverse transcription; PCR, polymerase chain reaction; PAUP, phylogenetic analysis using parsimony.

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In the invertebrates, on the other hand, only one gene is present that is considered the present-day representative of the ancestral peptide gene to the vasopressin/oxytocin superfamily (9). In the mollusc *Lymnaea stagnalis*, this gene encodes Lys-conopressin, a peptide that is structurally related to vasopressin and that has a wide distribution throughout the invertebrate phyla (9, 22–25). In Lys-conopressin, the chemical nature of the amino acid residue at position 8 is not important for peptide function, since replacement of this basic residue (lysine) by an aliphatic one (isoleucine) does not affect the potency of binding of the peptide to its receptor (9). These studies also show that Lys-conopressin, although structurally related to vasopressin, controls reproductive functions that are clearly analogous to the central and myoregulatory functions of oxytocin in vertebrate reproduction. In addition, Lys-conopressin serves vasopressin-like functions in the control of carbohydrate metabolism (26). Together, these observations suggest that functionally distinct vasopressin and oxytocin receptors may have evolved from a nondiscriminative Lys-conopressin receptor-like receptor by gene duplication and subsequent introduction of specificity determinants that enable discriminative binding of vasopressin and oxytocin on the basis of the chemical nature of amino acid residue 8.

We have recently cloned a Lys-conopressin receptor from the *Lymnaea* vas deferens, named *L. stagnalis* conopressin receptor (LSCPR), that displays discriminative binding properties with respect to residue 8 in Lys-conopressin (26). Here we report the cloning of a second Lys-conopressin receptor, named LSCPR2, that unlike the previously identified receptor (now renamed LSCPR1), does not discriminate between Lys-conopressin and its oxytocin-like synthetic analog Ile-conopressin. We show that vasopressin and oxytocin receptors may have evolved from an LSCPR2-like ancestral receptor, and propose a mechanism for the molecular evolution of specificity in the peptide-receptor pairs of the vasopressin/oxytocin superfamily. Finally, we discuss the role that preexisting receptor subtypes may have had in the historic development of functionally distinct vasopressin and oxytocin lineages.

MATERIALS AND METHODS

Animals and Peptides—Adult specimens of *L. stagnalis* (shell height 28–34 mm), bred in the laboratory under standard conditions (27), were used. Arg-vasopressin, Arg-vasotocin, Lys-vasotocin, oxytocin, mesotocin, and isotocin were obtained from Saxon Biochemicals (Hannover, Germany). Lys-conopressin and Arg-conopressin were obtained from Bachem Feinchemikalien AG (Budendorf, Switzerland). Ile-conopressin was synthesized as described previously (9).

Cloning and Sequencing of LSCPR2—Poly(A)$^+$ RNA was isolated from dissected vas deferens of *L. stagnalis* and reverse transcribed into oligo-(dT)$_{17}$-primed cDNA using 200 units of Molony murine leukemia virus reverse transcriptase (Life Technologies, Inc.). Two degenerate oligonucleotides, TM6s (5′-GGGAATTCG(G/T)N(A/G)(C/T)NTT(C/T)-N(C/T)N(A/C/T)TNTG(C/T)TGGN(C/T)NCC-3′), directed to a conserved region in TM6 of neuropeptide receptors in general, and TM7as (5′-CCGGATCC(A/G)TA(A/G/T)ATCCANGG(A/G)TT(A/G)CA(A/G)C-3′), directed to a conserved region in TM7 of vasopressin and oxytocin receptors, were used to amplify vasopressin and oxytocin receptor-like sequences. The primers contained at the 5′-end a recognition sequence for the restriction endonucleases *Eco*RI and *Bam*HI, respectively. PCR was performed in a 100-μl solution containing 10 animal equivalents of vas deferens cDNA, 200 μM of each of the four deoxynucleotides, 150 pmol of each of the primers TM6s and TM7as, 10 mM Tris-HCl (pH 8.4), 0.1 mg/ml gelatin, 50 mM KCl, 1.5 mM $MgCl_2$, and 2.5 units of *Taq* DNA polymerase (Perkin-Elmer Cetus). The PCR mixture was overlaid with 70 μl of mineral oil and incubated in a DNA thermal cycler (Perkin-Elmer Cetus) for 50 cycles of 1 min at 94 °C, 1 min at 50 °C, and 1 min at 72 °C. Amplified cDNA fragments were digested with *Eco*RI and *Bam*HI and separated on agarose gel, and fragments of the expected size were isolated, cloned into pBluescript (Stratagene), and sequenced. One fragment was used to screen several *Lymnaea* brain-specific λZAPII cDNA libraries in order to obtain a full-length clone. The insert-containing pBluescript phagemid corresponding to this clone (pBSCPR2) was rescued by *in vivo* excision and sequenced.

Functional Expression of LSCPR2 in Xenopus Oocytes—The protein-encoding region of pBSCPR2 was amplified using primers directed to the translation initiation and termination sequences of the cDNA. The primers contained at the 5′-end a recognition sequence for the restriction endonucleases *Bam*HI and *Eco*RI, respectively. The sense primer contained the Kozak consensus sequence for transcription initiation (CCACCATG; Ref. 28) to replace the *Lymnaea* sequence at this position. Amplification was carried out in a DNA thermal cycler using 3 units of Ultma DNA polymerase (Perkin-Elmer Cetus), under conditions recommended by the supplier. Reactions were incubated for 12 cycles of 15 s at 94 °C, 15 s at 60 °C, and 1.5 min at 72 °C. The cDNA fragment was digested with *Bam*HI and *Eco*RI, ligated into pGEMHE (29), and 5′-capped cRNA was transcribed *in vitro* from a 3′-end-linearized plasmid using 40 units of T7 RNA polymerase and Cap-Scribe transcription buffer (Boehringer Mannheim). After phenol extraction and ethanol precipitation, the cRNA was dissolved in bidistilled water, and 50 nl (~3 ng RNA) was injected into *Xenopus laevis* oocytes. After incubating the injected oocytes for 2–3 days at 20 °C (30), two-electrode voltage-clamped oocytes were challenged with 1.5-min applications of Lys-conopressin and various related peptides. Peptide-induced membrane currents were recorded at holding potentials of −50 mV. Dose-response curves were constructed by integrating the membrane currents over time and plotting these values (expressed as percentage of the maximal membrane current) semilogarithmically *versus* the ligand concentration. From the mean of three experiments the EC_{50} value was calculated.

RT-PCR—Male and female sex organs and glands, kidney, skin, brain, salivary gland, and hepatopancreas were dissected, and total RNA was isolated from each tissue in triplicate. Equal amounts of RNA (3–5 μg for each tissue) were reverse transcribed into oligo-(dT)$_{17}$-primed cDNA using 200 units of Molony murine leukemia virus reverse transcriptase (Life Technologies, Inc.), and of each reaction 5% was used directly in a PCR experiment. Primers specific for either LSCPR1 or LSCPR2 were used to amplify conopressin receptor cDNAs, and primers specific for *Lymnaea* fructose 1,6-biphosphate aldolase[2] were used as a positive control for each cDNA preparation. After 30 cycles of amplification, PCR products were separated on agarose gel, transferred to Hybond N$^+$ membrane (Amersham Corp.), hybridized to 5′-end ^{32}P-labeled primers specific for each product, and autoradiographed.

In Situ Hybridization—Digoxigenin-labeled run-off antisense RNA was synthesized from 5′-end-linearized plasmids pBSCPR1 (26) and pBSCPR2 using 20 units of T3 RNA polymerase and digoxigenin-UTP labeling mixture (both from Boehringer Mannheim). Hybridizations of alternately collected sections of *Lymnaea* brains were performed as described previously (26).

Phylogenetic Analysis—Sequences of the members of the vasopressin/oxytocin receptor family were extracted from a G protein-coupled receptor data base (31) and aligned using the program Clustal V (32). Alignments were adjusted manually, and the transmembrane domains, as defined by Baldwin (33), were utilized to generate a PAUP data matrix (34). An accepted mutation stepmatrix was calculated using the program AMP.[3] A branch and bound search was performed without the stepmatrix, and the three most parsimonious trees with tree lengths of 858 were selected. When the branch and bound search was repeated with the stepmatrix invoked, one of these trees was found to be the shortest. Using the PAUP program, 1,000 replicates of bootstrap analysis were performed using the heuristic search procedure. The calculated g_1 value of −0.869935 ($p > 0.01$) indicated that relevant phylogenetic information was contained within the data set (35).

RESULTS

Cloning of the LSCPR2 cDNA—PCR on vas deferens cDNA using primers TM6s and TM7as yielded four putative receptor cDNA fragments. Data base searches revealed that two fragments had significantly higher sequence similarity with the vertebrate vasopressin and oxytocin receptors than with any other G protein-coupled receptor. We recently reported the cloning of the full-length cDNA corresponding to the first fragment (26). Here, we used the second cDNA fragment to screen brain-specific λZAPII cDNA libraries for full-length clones.

[2] R. E. van Kesteren, unpublished results.
[3] Provided by K. A. Rice, Harvard University.

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A

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GCCAGGTCGGCTGACCAGCGCCTCGCTGTTATGCCAGGCATGCTATCATAGTCTGCTTGTATCTCAGCTGAATGGCTTCGACAATCCGACAACACCCTCAACGCGAAATGA  -43

CCCCTTCCTGAAAAGATTTCTCTTCTTTTTTTTTTTTCAAAC  ATG AGA CCG CCG ACA TCA GTA TTC ATT GCC AAT GTC TCA TCT GTT AAG GAC    51
                                            Met Arg Pro Pro Thr Ser Val Phe Ile Ala Asn Val Ser Ser Val Lys Asp    17
                                                                                     *
CCT ACC GCA GCG TCT CCC GAA ATC AAC TTA GCC GAC TGG AGG ATC GGA CAG GAC AAC GCC ACG AAT AAG ACG CTG ACC GGA AGG   135
Pro Thr Ala Ala Ser Pro Glu Ile Asn Leu Ala Asp Trp Arg Ile Gly Gln Asp Asn Ala Thr Asn Lys Thr Leu Thr Gly Arg    45
                                                                         *           *
GAC GAA AGT CTC GCG AGG ATT GAG ATC CTC GTC CAG AGC ATC ATC CTA GCC CTG GCG ATC ATC GGC AAC TCG TGC GTC CTC ACG   219
Asp Glu Ser Leu Ala Arg Ile Glu Ile Leu Val Gln Ser Ile Ile Leu Ala Leu Ala Ile Ile Gly Asn Ser Cys Val Leu Thr    73

GCA CTG GCC CGC CGC GGG AAG GCC GCG TCC AGG ATG CAC CTG TTC ATC TTC CAC CTG AGC ATC GCC GAC CTG CTG GTG GCC GTC   303
Ala Leu Ala Arg Arg Gly Lys Ala Ala Ser Arg Met His Leu Phe Ile Phe His Leu Ser Ile Ala Asp Leu Leu Val Ala Val   101

TTC AAC ATC CTG CCG CAG CTC ATC TGG GAC ATC ACG GAG CGG TTC TAC GGC GGG GAC CTG CTG TGC CGA TAC ATC AAG TTC ATG   387
Phe Asn Ile Leu Pro Gln Leu Ile Trp Asp Ile Thr Glu Arg Phe Tyr Gly Gly Asp Leu Leu Cys Arg Tyr Ile Lys Phe Met   129

CAG GTG TAC GTC ATG TAT CTC TCC ACG TAC ATG CTG GTG ATG ACC GCT GTG GAC AGG TAC CGC GCC GTC TGC CAC CCC CTC TCG   471
Gln Val Tyr Val Met Tyr Leu Ser Thr Tyr Met Leu Val Met Thr Ala Val Asp Arg Tyr Arg Ala Val Cys His Pro Leu Ser   157

GCC TTC AAC ACG TCC ACG CGC ACG CCC CTC TAC TGC ATG ATC GTC TCC GCC TAC GTC ATC TCC GGT GTC CTC AGC CTC CCG CAG   555
Ala Phe Asn Thr Ser Thr Arg Thr Pro Leu Tyr Cys Met Ile Val Ser Ala Tyr Val Ile Ser Gly Val Leu Ser Leu Pro Gln   185
         *
CCG ATC ATC TTC AAG TAC CGC GAG AAG AGC CAC GGC TCC GGA GAC TAC GAG TGC TGG GGC AGG TTC GAG CCG CCG TGG ACG CTG   639
Pro Ile Ile Phe Lys Tyr Arg Glu Lys Ser His Gly Ser Gly Asp Tyr Glu Cys Trp Gly Arg Phe Glu Pro Pro Trp Thr Leu   213

AAC CTG TAC ATC ACC TCG TTC ACG TTC GCC GTG TAC ATC GTG CCT CTG GCC ATA TTG ATA TTC GCG TAC GTC TCC ATA TGC TGC   723
Asn Leu Tyr Ile Thr Ser Phe Thr Phe Ala Val Tyr Ile Val Pro Leu Ala Ile Leu Ile Phe Ala Tyr Val Ser Ile Cys Cys   241

ACG ATA TGG AGG AAA TAC AAG AGC GCG GAG AAC GAG CGG AAG CAC ATG CTG AAC GGC TCG GAC TCG TCC CTC GGG AAC AGG AAC   807
Thr Ile Trp Arg Lys Tyr Lys Ser Ala Glu Asn Glu Arg Lys His Met Leu Asn Gly Ser Asp Ser Ser Leu Gly Asn Arg Asn   269
                                                                     *
ATC TAC AGC AAC CAC GTG ACC CAC TCG GCG CTG TTC AGG CAC AGG GGC GTC ATC GAG AGG AGG CGG AAT CTC GTG CAG CGG TGC   891
Ile Tyr Ser Asn His Val Thr His Ser Ala Leu Phe Arg His Arg Gly Val Ile Glu Arg Arg Arg Asn Leu Val Gln Arg Cys   297

AGG CCG GCG CCG ATG GCA GCC CCG AGG GCG CAC AGT CTG AGG GGG TTT TCC AGG GCC AAG TTG AAG ACG GTG AAG CTG ACG TTT   975
Arg Pro Ala Pro Met Ala Ala Pro Arg Ala His Ser Leu Arg Gly Phe Ser Arg Ala Lys Leu Lys Thr Val Lys Leu Thr Phe   325
                                             •                                   •
GTG GTG ATT GTG GCG TAC GTG GTG TGC TGG TCG CCG TTC TTT CTG TCG CAG CTG TGG TGG CTG TAC GAC GAG CAG CAG GAG CAC  1059
Val Val Ile Val Ala Tyr Val Val Cys Trp Ser Pro Phe Phe Leu Ser Gln Leu Trp Trp Leu Tyr Asp Glu Gln Gln Glu His   353

AAT CAT GCA GTG GTC ATC ATG GTG CTC TTG GCA AGT CTC AAC AGC TGC TGC AAT CCA TGG ATA TAT CTG GCC TTT AGC GGT AAC  1143
Asn His Ala Val Val Ile Met Val Leu Leu Ala Ser Leu Asn Ser Cys Cys Asn Pro Trp Ile Tyr Leu Ala Phe Ser Gly Asn   381

CTG ATC AGA CAC ATC TGT CCC ATG AGG CCA AAC AGC AGA AAT TGT TGT TGC TGC GAT TTG CGT CGG TGC CGC CGC CGT CAG TTT  1227
Leu Ile Arg His Ile Cys Pro Met Arg Pro Asn Ser Arg Asn Cys Cys Cys Cys Asp Leu Arg Arg Cys Arg Arg Arg Gln Phe   409

TAC CGT CGG AGA AGC AGC AGC AAC AAC AAC CAC GAT GAC ACC CAC AGT CCG CTG TCA CCG TCG GAC GAG AGG ATT CAC CAC CAC  1311
Tyr Arg Arg Arg Ser Ser Ser Asn Asn Asn His Asp Asp Thr His Ser Pro Leu Ser Pro Ser Asp Glu Arg Ile His His His   437
                 †   †                                                   Δ
AGG GAT GGT ATG CTG GAT GAT ATG GAA CTA AGG GAA CTT ATC GCT GAT GGA ATC AAC CGT AAG CGC CAG CCT TAC ATC AAG TCG  1395
Arg Asp Gly Met Leu Asp Asp Met Glu Leu Arg Glu Leu Ile Ala Asp Gly Ile Asn Arg Lys Arg Gln Pro Tyr Ile Lys Ser   465
                                                                                                             Δ
GCG CCC GAC ACC GTC ACG TCC AAG ACC GTC ACC ACC AAA ACT GGG ACC CCC ATC AGC AGC ACC CAG CTG GTG AAG AGA GAA TCG  1479
Ala Pro Asp Thr Val Thr Ser Lys Thr Val Thr Thr Lys Thr Gly Thr Pro Ile Ser Ser Thr Gln Leu Val Lys Arg Glu Ser   493
                     •                   •                                                                   †
CTG CAC AAG ACG GCC GTC CTT CAG AAC GGG AGA CAC GGC CTT GGG CTG GAC TGG ACA CAC CCG CTT CCC AAC AAT CTG GTC TGA  1563
Leu His Lys Thr Ala Val Leu Gln Asn Gly Arg His Gly Leu Gly Leu Asp Trp Thr His Pro Leu Pro Asn Asn Leu Val End   520
```

B

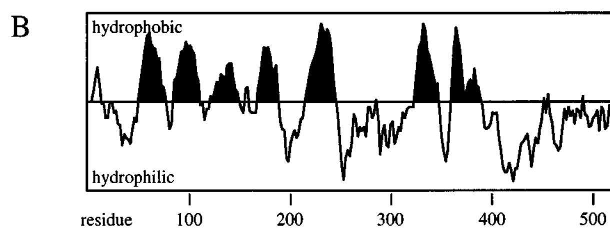

FIG. 1. **The primary structure of LSCPR2.** *A*, nucleotide sequence and deduced amino acid sequence of clone pBSCPR2. Only the open reading frame and a part of the 5′-untranslated leader sequence are shown. Nucleotides and amino acids are *numbered* at the right hand side, starting at the first ATG and the corresponding Met residue. Nucleotides upstream from the first ATG are indicated with negative numbers. An in-frame stop codon at position −45 is *underlined*. Putative transmembrane domains in the predicted protein sequence are shown in *reversed contrast*. Putative sites for *N*-linked glycosylation (Asn-*X*-Ser/Thr) are indicated by *asterisks*, and putative sites for phosphorylation by protein kinase C (Ser/Thr-*X*-Arg/Lys), casein kinase II (Ser/Thr-*X*-*X*-Asp/Glu) and cAMP-dependent protein kinase (Arg/Lys-Arg/Lys-*X*-Ser/Thr) are indicated by *dots*, *triangles*, and *daggers*, respectively. *B*, hydrophobicity plot of the LSCPR2 protein according to Kyte and Doolittle (36). *Numbers* below the figure indicate amino acid residue positions in the LSCPR2 protein. The seven hydrophobic regions that represent putative transmembrane domains are shown in *black*.

From 4×10^6 independent clones that were screened, one clone hybridized and was isolated. The corresponding pBluescript phagemid, named pBSCPR2, was rescued by *in vivo* excision and sequenced from both strands. pBSCPR2 has an insert of 4.5 kilobase pairs and contains an open reading frame of 1,560 base pairs encoding a 520-amino acid protein with a predicted molecular mass of 59.4 kDa (Fig. 1*A*). The open reading frame is preceded by an in-frame stop codon at position −45, which indicates that the coding region is complete at the 5′-end.

Structural Characteristics of LSCPR2—Hydrophobicity analysis (36) of the predicted amino acid sequence revealed 7 hydrophobic domains characteristic for the 7 TMs of G protein-coupled receptors (Fig. 1*B*; Ref. 33). Therefore, and because of its sequence similarity with vasopressin and oxytocin receptors and with LSCPR1, the predicted protein was tentatively named *L. stagnalis* conopressin receptor 2 (LSCPR2). LSCPR2 contains several potential sites for *N*-linked glycosylation and for phosphorylation (Fig. 1*A*). Consensus sequences for *N*-glycosylation (Asn-*X*-Ser/Thr; Ref. 37) occur predominantly in the extracellular N-terminal domain and may have a function in targeting the receptor protein to the plasma membrane (38). Consensus sequences for phosphorylation sites (39) include four sites for protein kinase C (Ser/Thr-*X*-Arg/Lys), two sites for casein kinase II (Ser/Thr-*X*-*X*-Asp/Glu), and three sites for cAMP-dependent protein kinase (Arg/Lys-Arg/Lys-*X*-Ser/Thr). These are restricted to the third intracellular loop (*i.e.* between TM5 and TM6) and the C terminus, suggesting that phosphorylation may be important in the modulation of G protein coupling and receptor function (1).

An amino acid sequence alignment of LSCPR2, LSCPR1, and

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                                                                                                            TM I
LSCPR1  MDLELNLTNGFMLMPPTKRAVIQIASNEKVGYDVSKSQPVNATTDLNTTRMNATNSTEVSYFHGVDEDLLKIEIAVQATILYMTLFGNGIVL
LSCPR2                      MRPPTSVFIANVSSVKDPTAASPEINLADWRIGQDNATNKTLT----GRDESLARIEILVQSIILALAIIGNSCVL
HSOTR                                      MEGALAANWSAEAANASAAPPGAEGNRTAGPP--RRNEALARVEVAVLCLILLLALSGNACVL
RNAV1a                         MSFPRGSQDRSVGNSSPWWPLTTEGSNGSQEAARLGEGDSPLGDVRNEELAKLEIAVLAVIFVVAVLGNSSVL
HSAV1b                                          MDSGPLWDANPTPRGTLSAPNATTPWL--GRDEELAKVEIGVLATVLVLATGGNLAVL
CCVTR                                                   MGRIANQTTASNDTDPF---GRNEEVAKMEITVLSVTFFVAVIGNLSVL
RNAV2                                           MLLVSTVSAVPGLFSPPSSPSNSSQEELLDDRDPLLVRAELALLSTIFVAVALSNGLVL

                          TM II                                  TM III
LSCPR1  LVL-RLRRQKL-TRMQWFIAHLAFADIFVGFFNILPQLISDVTIVFHGDDFTCRFIKYFQVIAMYASSYVLVMAAIDRYLSICHPLTSQTLS
LSCPR2  TAL-ARRGKAA-SRMHLFIFHLSIADLLVAVFNILPQLIWDITERFYGGDLLCRYIKFMQVYVMYLSTYMLVMTAVDRYRAVCHPLSAFNTS
HSOTR   LAL-RTTRQKH-SRLFFFMKHLSIADLVVAVFQVLPQLLWDITFRFYGPDLLCRLVKYLQVVGMFASTYLLLLMSLDRCLAICQPLRSLRRR
RNAV1a  LAL-HRTPRKT-SRMHLFIRHLSLADLAVAFFQVLPQLCWDITYRFRGPDWLCRVVKHLQVFAMFASAYMLVVMTADRYIAVCHPLKTLQQP
HSAV1b  LTL-GQLGRKR-SRMHLFVLHLALTDLAVALFQVLPQLLWDITYRFQGPDLLCRAVKYLQVLSMFASTYMLLAMTLDRYLAVCHPLRSLQQP
CCVTR   LAM-HNTKKKS-SRMHLFIKHLSLADMVVAFFQVLPQLCWEITFRFYGPDFLCRIVKHLQVLGMFASTYMMVMMTLDRYIAICHPLKTLQQP
RNAV2   GALIRRGRRGRWAPMHVFISHLCLADLAVALFQVLPQLAWDATDRFHGPDALCRAVKYLQMVGMYASSYMILAMTLDRHRAICRPMLAYRHG
                                          *******        * ***

                  TM IV                                               TM V
LSCPR1  PKRV-HLMIALAWLISFLCALPQVFIFSLQAVGPD----QYDCLATFEPDWGMQAYITWVFVANYVIPFLLLAFCYGRICHVVWMSVAAKES
LSCPR2  TRTPLYCMIVSAYVISGVLSLPQPIIFKYREKSHGSG--DYECWGRFEPPWTLNLYITSFTFAVYIVPLAILIFAYVSICCTIWRKYKSAEN
HSOTR   TD---RLAVLATWLGCLVASAPQVHIFSLREVADG----VFDCWAVFIQPWGPKAYITWITLAVYIVPVIVLATCYGLISFKIWQNLRLKTA
RNAV1a  ARRS-RLMIATSWVLSFILSTPQYFIFSVIEIEVNNGTKTQDCWATFIQPWGTRAYVTWMTSGVFVAPVVVLGTCYGFICYHIWRNIRGKTA
HSAV1b  GQST-YLLIAAPWLLAAIFSLPQVFIFSLREVIQGSG--VLDCWADFGFPWGPRAYLTWTTLAIFVLPVTMLTACYSLICHEICKNLKVKTQ
CCVTR   TQRA-YIMIGSTWLCSLLLSTPQYFIFSLSEIQNGSY--VYDCWGHFIEPWGIRAYITWITVGIFLIPVIILMICYGFICHSIWKNIKCKTM
RNAV2   GGARWNRPVLVAWAFSLLLSLPQLFIFAQRDVGNGSG--VFDCWARFAEPWGLRAYVTWIALMVFVAPALGIAACQVLIFREIHASLVPG--
                                                    **** *  ***
                                                                                          TM VI
LSCPR1  AAYSSMRNGCTESSRPIKMRISFHRRRDNTNATLTSLDRHDASAVTSSDSKKPRGHQ-RGVSKSKMKTIKLTLTVVLCYLFCWAPFFVVQMW
LSCPR2  ERKHMLNGSDSSLGNRNIYSNHVTHSALFRHRGVIERRRNLVQRCRPAPMAAPRAHSLRGFSRAKLKTVKLTFVVIVAYVVCWSPFFLSQLW
HSOTR   A-----------------------------AAAAEAPEGAAAGDGGRVALARVSSV-KLISKAKIRTVKMTFIIVLAFIVCWTPFFFVQMW
RNAV1a  S-------------------------SRHSKGDKGSGEAVGPFHKGLLVTPCVSSV-KSISRAKIRTVKMTFVIVSAYILCWAPFFIVQMW
HSAV1b  A----------------------WRVGGGGWRTWDRPSPSTLAATTRGLPSRVSSI-NTISRAKIRTVKMTFVIVLAYIACWAPFFSVQMW
CCVTR   ------------------------------------RGTRNTKDGMIGKVSVSSV-TIISRAKLRTVKMTLVIVLAYIVCWAPFFIVQMW
RNAV2   ------------------------------------PSERAGTPQRAPDRSPSEG-AHVSAAMAKTVRMTLVIVIVYVLCWAPFFLVQLW

                  TM VII
LSCPR1  SAFDDD---SGIEHPVTVICMLLASLNSCTNPWIYLAFSGRTCQRNHANRNTSRSWGPSTHVTSGGDSHEFKTRSSFIDLSHARMNARSSSP
LSCPR2  WLYDEQ----QEHNHAVVIMVLLASLNSCCNPWIYLAFSGNLIRHICPMRPNSRNCCCCDLRRCRRRQFYRRRSSSNNNHDDTHSPLSPSDE
HSOTR   SVWDAN---APKEASAFIIVMLLASLNSCCNPWIYMLFTGHLFHELVQRFLCCSASYLKGRRLGETSASKKSNSSSFVLSHRSSSQRSCSQP
RNAV1a  SVWDENFIWTDSENPSITITALLASLNSCCNPWIYMFFSGHLLQDCVQSFPCCHSMAQKFAKDDSDSMSRKTDFLF
HSAV1b  SVWDKNAPDEDSTNVAFTISMLLGNLNSCCNPWIYMGFNSHLLPRPLRHLACCGGPQPRMRRRLSDGSLSSRHTTLLTRSSCPATLSLSLSL
CCVTR   SVWDENFSWDDSENAAVTLSALLASLNSCCNPWIYMLFSGHLLYDFLRCFPCCKKPRNMLQKEDSDSSIRRNTLLTKLAAGRMTNDGFGSWR
RNAV2   AAWDPE---APLERPPFVLLMLLASLNSCTNPWIYASFSSSVSSE-LRSLLCCAQRHTTHSLGPQDESCATASSSLMKDTPS

LSCPR1  RFSEMNKLNHSKC
LSCPR2  RIHHHRDGMLDDMELRELIADGINRKRQPYIKSAPDTVTSKTVTTKTGTPISSTQLVKRESLHKTAVLQNGRHGLGLDWTHPLPNNLV
HSOTR   STA
RNAV1a
HSAV1b  TLSGRPRPEESPRDLELADGEGTAETIIF
CCVTR   DPCNSRKSSQSIGLDCFCKSSQCLEHDCSRKSSQCIPLDCSRKSSQCIPLDCSRKSSQCMSKES
RNAV2
```

FIG. 2. **Alignment of the amino acid sequences of LSCPR2 and members of the vasopressin/oxytocin receptor family.** The amino acid sequence of LSCPR2 is aligned with those of *LSCPR1* (26), the human oxytocin receptor (*HSOTR*; Ref. 20), the rat V1a vasopressin receptor (*RNAV1a*; Ref. 17), the human V1b vasopressin receptor (*HSAV1b*; Ref. 19), the vasotocin receptor of the teleost fish *Catostomus commersoni* (*CCVTR*; Ref. 41), and the rat V2 vasopressin receptor (*RNAV2*; Ref. 14). Amino acid residues that are identical in all receptor proteins are shown in *boldface*. *Asterisks* indicate amino acid residues that are highly conserved in the vertebrate vasopressin/oxytocin receptor family, but not in other G protein-coupled receptors and have been suggested to be important in receptor-ligand interaction (6). *Bars* indicate the seven putative transmembrane domains.

receptors for vasopressin, oxytocin, and vasotocin from vertebrates is presented in Fig. 2. In the region from the beginning of TM1 to the end of TM7, LSCPR2 has 43% sequence identity with the human oxytocin receptor, the rat V1a, and the human V1b vasopressin receptors and the fish vasotocin receptor, and 39% sequence identity with LSCPR1 and the rat V2 vasopressin receptor. Sequence identity is highest in the transmembrane domains, especially in TM7, whereas the N- and C-terminal domains as well as the third intracellular loop show hardly any sequence identity.

Several amino acid residues (indicated by *asterisks* in Fig. 2) that are conserved only among the members of the vasopressin/oxytocin receptor family are thought to be important in ligand binding (6). In LSCPR2, these residues are either identical or conserved, with the exception of a threonine residue replacing the glycine residue in the sequence Pro-Trp-Gly (just before TM5). Interestingly, the two conserved proline residues in the sequences Gly-Pro-Asp and Pro-Trp-Gly (just before TM3 and TM5, respectively), which are replaced by aspartate residues in LSCPR1, are unchanged in LSCPR2.

Two adjacent cysteines that are found 15 residues downstream of TM7 in the vasopressin and oxytocin receptors may be responsible for anchoring the C terminus to the plasma membrane through palmitoylation, as has been observed for the β-adrenergic receptor (40). In LSCPR2, there are 4 adjacent cysteines present 4 residues further downstream. These may be involved in linking the C terminus of the receptor to the plasma membrane through a palmitoylation anchor.

Functional Expression of LSCPR2 in Xenopus Oocytes—To demonstrate that LSCPR2 indeed is a receptor for Lys-conopressin, we have expressed the LSCPR2 cDNA in *Xenopus* oocytes by injection of cRNA and studied the response of injected oocytes upon application of Lys-conopressin and related peptides. To improve cRNA stability and expression levels in *Xenopus* oocytes, the open reading frame of LSCPR2 was cloned in pGEMHE, between the 5′- and 3′-untranslated sequences of the *Xenopus* β-globin gene (29). Voltage-clamped oocytes that were previously injected with 5′-capped cRNA derived from this construct responded to application of conopressins by displaying inward membrane currents (Fig. 3*A*). Only application of Lys-conopressin and its synthetic oxytocin-like analog Ile-conopressin resulted in maximal responses. Arg-conopressin was about 10 times less potent, and all of the vertebrate peptides tested had no effects at concentrations as high as 10^{-6} M. The effects of Lys-conopressin and Ile-conopressin were dose-dependent, with estimated EC_{50} values of 86 and 96 nM, respectively (Fig. 3*B*). These values are about four times higher than observed for LSCPR1 (26).

Differential Expression of the LSCPR1 and LSCPR2 Genes—Since LSCPR1 and LSCPR2 mRNA levels are undetectable using Northern blot analysis (data not shown), we applied RT-PCR to determine possible co-expression of the LSCPR1 and LSCPR2 genes in peripheral tissues and brain. The expression of both receptor genes is restricted to the brain and the reproductive organs (Fig. 4). Interestingly, the anterior part of the vas deferens contains exclusively LSCPR1 mRNA, whereas the spermoviduct contains exclusively LSCPR2 mRNA. In the posterior part of the vas deferens, a small amount of both transcripts was detected. *In situ* hybridization on alternate sections of the brain revealed that the two receptor genes are expressed in mutually exclusive sets of neurons. As reported previously (26), the LSCPR1 gene is expressed in neurons in the anterior lobe of the right cerebral ganglion and the pedal Ib cluster, in a subpopulation of the neuroendocrine light green cells, and in some unidentified neurons throughout the brain (not shown). The LSCPR2 gene is predominantly expressed in neurons in the visceral ganglion (Fig. 5*A*) and the right parietal ganglion (Fig. 5*B*) and in some neurons that are located in close proximity of the anterior lobes of the cerebral ganglia (Fig. 5*C*). Control hybridizations with sense cRNA probes were all negative.

Phylogenetic Relationships of Lys-Conopressin Receptors and Their Vertebrate Counterparts—From a sequence alignment of the transmembrane domains of the members of the vasopressin/oxytocin receptor family, a most parsimonious phylogenetic tree was calculated. This tree (Fig. 6) has an unweighted tree

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FIG. 3. **Functional expression of LSCPR2 in *Xenopus* oocytes.** *A*, membrane current traces of an oocyte expressing the LSCPR2 protein and being exposed to 10^{-6} M vasopressin, oxytocin, Arg-conopressin, Lys-conopressin, and Ile-conopressin (indicated by *bars*). Only application of conopressins elicited membrane current responses, whereas vasopressin and oxytocin as well as all the other vertebrate peptides tested were inactive. The initial fast inward current was typically followed by an oscillatory current, which represents intracellular calcium oscillations and lasted for about 5 min after washing out of the peptide. *B*, dose-response curves of the effects of Lys-conopressin (*black circles*) and Ile-conopressin (*open squares*) on the total membrane current. The total membrane currents elicited by 1.5-min applications of either of the peptides at different concentrations were integrated over time and plotted semilogarithmically *versus* the peptide concentration. The mean of three independent experiments is shown. The EC_{50} values are 86 nM for Lys-conopressin and 96 nM for Ile-conopressin.

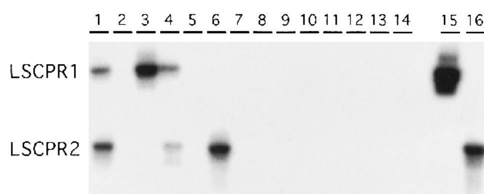

FIG. 4. **Tissue distribution of LSCPR1 and LSCPR2 as determined by RT-PCR.** LSCPR1 and LSCPR2 expression were analyzed in brain (*lane 1*), penis complex (*lane 2*), anterior vas deferens (*lane 3*), posterior vas deferens (*lane 4*), prostate gland (*lane 5*), spermoviduct (*lane 6*), ovotestis (*lane 7*), albumen gland (*lane 8*), oothecal gland (*lane 9*), muciparous gland (*lane 10*), kidney (*lane 11*), skin (*lane 12*), salivary gland (*lane 13*), and hepatopancreas (*lane 14*). The LSCPR1 transcript could be amplified from the brain and the anterior and posterior vas deferens, and the LSCPR2 transcript could be amplified from the brain, the spermoviduct and the posterior vas deferens. *Lanes 15* and *16* contain PCR products derived from the LSCPR1 and LSCPR2 clones, respectively, and serve as positive controls for the PCR and the hybridization procedure.

length of 858 and is the best tree found in over 327,128 trees sampled. It demonstrates that LSCPR1 and LSCPR2 are clearly related to the vasopressin/oxytocin receptor family of the vertebrates. Bootstrap analysis of the tree and the data set show that the assignment of LSCPR1 and LSCPR2 to the vasopressin/oxytocin receptor clade is supported with over 96% confidence. These findings strongly suggest that the conopressin receptors and their vertebrate counterparts evolved from a common ancestral receptor. Moreover, the tree shows that LSCPR2 is more closely related to the vertebrate receptors than LSCPR1 and that the separate evolutionary history of LSCPR1 and LSCPR2 is probably much longer than that of the vertebrate receptor subtypes.

DISCUSSION

Structure and Functional Expression of LSCPR2—To provide molecular data for a theory of the co-evolution of peptide-receptor pairs, we have cloned various Lys-conopressin receptors in the mollusc *L. stagnalis*. Using degenerate oligonucleotides directed to conserved sequences in the mammalian vasopressin and oxytocin receptors, we amplified similar sequences from the vas deferens of *Lymnaea*. The cloning of the full-length cDNA corresponding to one of these amplification products was recently reported (26). Here, we have used a second amplification product exhibiting high sequence identity with vertebrate vasopressin and oxytocin receptors in order to obtain a full-length cDNA. Hydrophobicity analysis of the predicted protein sequence of this clone, named LSCPR2, revealed that it contains seven hydrophobic regions similar to the seven transmembrane domains found in all members of the G protein-coupled receptor superfamily. In addition, LSCPR2 contains amino acid residues in the first and second extracellular loops that are characteristic for the members of the vasopres-

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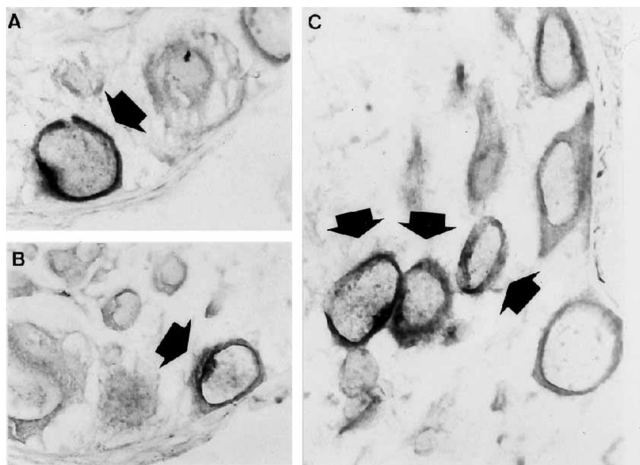

FIG. 5. **Localization of LSCPR2 gene expression in the brain by *in situ* hybridization.** *A*, section through the visceral ganglion; *B*, section through the right parietal ganglion showing two unidentified neurons, indicated by *arrows*, that express the LSCPR2 gene; *C*, section through the right cerebral ganglion ganglion showing a small group of unidentified neurons, indicated by *arrows*, that expresses the LSCPR2 gene. Neither of these neurons was found to express the LSCPR1 gene (not shown), nor could co-expression of LSCPR1 and LSCPR2 be detected in any other part of the brain. (Magnification, 250X.)

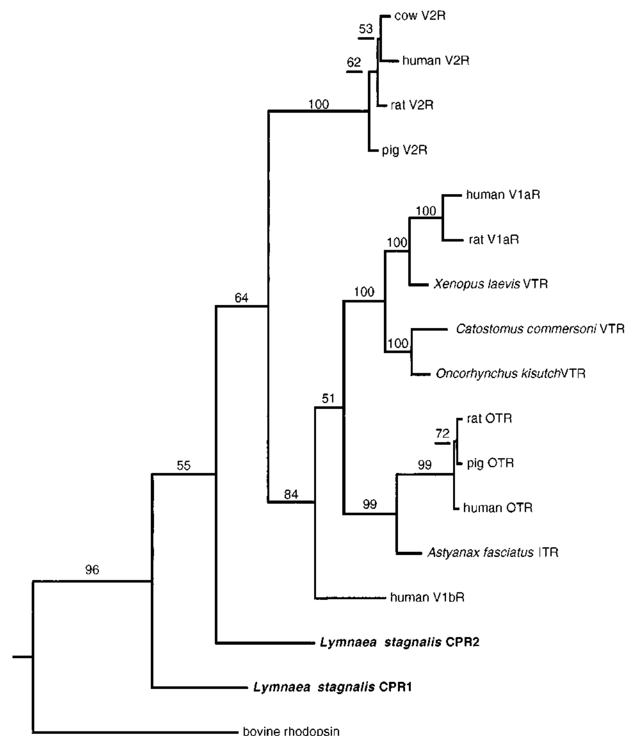

FIG. 6. **Phylogenetic relationships of the members of the vasopressin/oxytocin receptor family.** Using an accepted mutation methodology (L. F. Kolakowski, Jr., and K. A. Rice, submitted for publication), the phylogenetic relationships of LSCPR1, LSCPR2, and related receptors from vertebrates were analyzed. The most parsimonious tree resulting from these analyses is shown. *Numbers* above the branches are the results of a bootstrap analysis and are confidence limits for the positions of the branches. The abbreviations used are as follows: *V2R*, V2 vasopressin receptor; *V1aR*, V1a vasopressin receptor; *V1bR*, V1b vasopressin receptor; *VTR*, vasotocin receptor; *OTR*, oxytocin receptor; *ITR*, isotocin receptor; *CPR*, conopressin receptor. The vasotocin receptors from the toad *X. laevis* and from the teleost fish *Oncorhynchus kisutch*, as well as the isotocin receptor from the teleost fish *Astyanax fasciatus*, have been cloned partially (41) and are classified as such on the basis of their positions in the tree. The *Bovine rhodopsin* sequence was utilized as an outgroup for the tree.

sin/oxytocin receptor family (Fig. 2). LSCPR2 is activated by Lys-conopressin at physiological concentrations, but not by other naturally occurring vasopressin- or oxytocin-related peptides (Fig. 3). Together, these data indicate that LSCPR2 is a functional Lys-conopressin receptor, and that LSCPR1 and LSCPR2 represent distinct subtypes of Lys-conopressin receptors in *Lymnaea*.

Compared with the vertebrate receptors, the LSCPR2 protein has the highest sequence identity with the mammalian V1a and V1b vasopressin receptors (17, 19), the mammalian oxytocin receptor (20), and the vasotocin receptor from teleost fish (41), which all couple to the inositol trisphosphate/Ca^{2+} signaling pathway. Sequence identity with the mammalian V2 vasopressin receptor (13, 14), which is coupled to adenylate cyclase, is less. Furthermore, *Xenopus* oocytes expressing the LSCPR2 protein respond to application of Lys-conopressin by displaying a dose-dependent inward chloride current (Fig. 3), a response that is characteristic for receptors that activate the inositol trisphosphate/Ca^{2+} signal transduction pathway (30). These findings indicate that most likely LSCPR2 is coupled to a G protein that activates the inositol trisphosphate/Ca^{2+} signal transduction pathway. LSCPR1 has similar signal transduction characteristics, however, half-maximal receptor activation is reached at four times lower concentrations of Lys-conopressin (26). The first and second extracellular loops of the vasopressin and oxytocin receptors have been suggested to form a ligand binding pocket (6). Interestingly, the corresponding loops in LSCPR1 contain two unique aspartate residues that are absent in LSCPR2 (Fig. 2). Since conopressins differ from other vasopressin- and oxytocin-related peptides in that they have a negatively charged arginine residue at position 4 (22), we have suggested that either of these positively charged aspartate residues may be important in the interaction of LSCPR1 with this arginine residue (26). The absence of the aspartate residues in LSCPR2 might explain the fact that this receptor is less sensitive to Lys-conopressin than LSCPR1.

Differential Expression of Conopressin Receptor Subtypes in Lymnaea—To investigate the physiological significance of multiple conopressin receptors in *Lymnaea*, we compared the peripheral and neuronal distribution of LSCPR1 and LSCPR2 by means of RT-PCR and *in situ* hybridization. The expression of both receptors is restricted to the reproductive organs and the brain. LSCPR1 is exclusively expressed in the vas deferens (Fig. 4) and is involved in the control of muscular activity during transport of semen (26). LSCPR2 is predominantly expressed in the spermoviduct, and a small amount of transcript could be detected in the posterior part of the vas deferens (Fig. 4). In the spermoviduct, both eggs and sperm are transported, and fertilization of eggs and resorption of unused sperm occur. Interestingly, Lys-conopressin mRNA could also be detected in this tissue,[4] suggesting that LSCPR2 might mediate auto- or paracrine actions of Lys-conopressin in the control of any of the reproductive functions of the spermoviduct. Similarly, vasopressin has been suggested to have an autocrine regulatory role in Leydig cells of rodent testis (42). Although LSCPR1 and LSCPR2 transcripts are co-localized in the posterior part of the vas deferens, the LSCPR1 mRNA detected here is probably synthesized in central neurons that innervate the vas deferens (26). The cellular source of LSCPR2 mRNA in the vas deferens remains unclear. In the brain, co-localization of LSCPR1 and LSCPR2 transcripts could not be observed.

The differential distribution of the two Lys-conopressin receptors suggests that they must have different functions. In the periphery, they are most likely involved in the regulation of distinct aspects of reproduction, whereas in the brain, they

[4] R. E. van Kesteren, unpublished results.

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probably mediate transmitter-like or modulatory effects of Lys-conopressin on different types of neurons. A possible explanation for the existence of multiple conopressin receptors is that it allows a stimulus-dependent differential expression of the receptor genes in various target tissues and cells, thus providing a mechanism for a spatio-temporal co-ordination of Lys-conopressin actions in otherwise conflicting types of behavior and physiological processes. Similarly, transcriptional regulation of the human oxytocin receptor gene plays an important role in the physiologically relevant increase in the number of oxytocin receptors in the uterus of pregnant females at the onset of labor (20). In addition, the difference in the sensitivity of the two receptors to Lys-conopressin that we observed may be physiologically relevant in this respect as well.

Prospects for Understanding the Co-evolution of Specifically Interacting Peptide-Receptor Pairs—Peptides are extensively employed as messenger molecules in the various communication systems that are involved in the control of physiological processes and behavior (43). These systems, which include wiring transmission (communication via the synapse), volume transmission (communication via the intercellular space), hormonal transmission (communication via the circulation), and environmental transmission, emerged at various stages during evolution. Due to differences in requirements for optimized information exchange, both peptide design and specificity of peptide-receptor interactions are under different selection pressures in the various communication channels (43, 44). With the evolution of complex organisms such as vertebrates, many peptides and receptors were recruited into the hormonal communication channel, thus increasing the demands for specificity in order to exclude cross-activation of the many different receptors that can be reached. In view of the great variety in both size and conformational flexibility of peptides, it seems plausible that different structural solutions emerged in response to the need for specificity of peptide-receptor interactions. Large and flexible peptides such as luteinizing hormone and follicle-stimulating hormone, which like vasopressin and oxytocin evolved from a single ancestral gene, have acquired distinct regions of many amino acid residues that exclude cross-activation of receptors (45). Consequently, these specificity determinants are mirrored in the luteinizing hormone and follicle-stimulating hormone receptors at entirely different locations.

As we have shown, specificity determinants in the small and conformationally constrainted peptides of the vasopressin/oxytocin superfamily evolved in a different way. Residues 1–6 of these peptides are important for high affinity binding to the receptor (26), whereas specific receptor activation depends on the chemical nature of the single amino acid residue at position 8. In contrast to the development of luteinizing hormone and follicle-stimulating hormone and their receptors, where separation of large specificity domains in both the peptides and the receptors allowed for a continuous refinement of specificity during evolution (45), functionally distinct vasopressin and oxytocin peptides can only have evolved by trial-and-error mutation of residue 8 in the presence of a nondiscriminative receptor such as LSCPR2. This trial-and-error phase may be reflected in various present day species of cartilaginous fish. Unlike the evolutionary stable vasopressin lineage, oxytocin-like peptides are very diverse in this primitive group of the vertebrates (7). To none of these peptides has a function been assigned, and they have very poor uterotonic activity in mammals (46). Hence, it will be of interest to examine whether functional receptors exist for these peptides. If not, they must be considered relicts of an early step in the historic development of the oxytocin lineage of bioactive peptides.

To further demonstrate that a nondiscriminative LSCPR2-like receptor may have been ancestral to the vasopressin/oxytocin receptor family, we studied the phylogenetic relationships of the Lys-conopressin receptors and their vertebrate counterparts using parsimony analysis. Phylogenetic reconstruction is nowadays accepted as a valid method to study the evolutionary histories of families of related receptors and provides useful insights into the structures and functions of the individual members (47).[5] Our data indicate that LSCPR2 is indeed more closely related to the vertebrate vasopressin and oxytocin receptors than LSCPR1 (Fig. 6). Therefore, LSCPR2 most likely is a present day representative of the ancestral receptor to the vertebrate receptors. In addition, the phylogenetic analysis shows that LSCPR1 and LSCPR2 probably result from an ancient receptor duplication that occurred before separate receptor types in the vertebrates evolved. Thus, multiple receptors may have existed before separate lineages of vasopressin- and oxytocin-related peptides evolved. Since the evolution of functionally distinct peptide lineages from a common ligand-receptor pair requires not only the introduction of specificity determinants on both the peptides and the receptors but also a differential cellular pattern of expression of distinct receptor types, we suggest that preexistence of differentially expressed receptor subtypes was of significant importance in the functional divergence of vasopressin and oxytocin in the vertebrates.

As in the peptides of the vasopressin/oxytocin superfamily, specificity determinants in the corresponding receptors are probably restricted to small parts of the receptor molecules (6). LSCPR2 can be very useful in the search for these determinants. Experiments involving chimeric receptors and swapping of putative binding domains might mimmick the evolutionary process of introducing specificity determinants in a nondiscriminative ancestral receptor such as LSCPR2, and increase our understanding of the molecular basis of peptide-receptor interactions. This then might enable the rational design of highly potent and specific receptor agonists and antagonists.

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