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Molecular Cloning and Pharmacological Characterization of a Molluscan Octopamine Receptor

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SUMMARY

We describe the cloning and functional expression of a cDNA encoding a novel G protein-coupled receptor, which was isolated from the central nervous system of the pond snail *Lymnaea stagnalis*. The amino acid sequence predicted by this cDNA shows highest similarity with the sequence of the *Locusta* tyramine receptor, the *Drosophila* tyramine/octopamine receptor, and the mammalian α-adrenergic receptors. On expression in mammalian cells, [^{3}H]rauwolscine, an α_2-adrenergic receptor antagonist, binds with high affinity ($K_D = 2.9 \times 10^{-9}$ M) to the receptor. Of several tested neurotransmitters, octopamine (which is considered to be the invertebrate counterpart of norepinephrine) showed the highest affinity (1.9×10^{-6} M) for the receptor. Therefore, we consider this receptor to be the first true octopamine receptor to be cloned. The ligand binding properties of the novel receptor, designated Lym oa$_1$, seem to be distinct from any of the binding profiles described for octopamine receptors in tissue preparations. Although the pharmacological profile of Lym oa$_1$ shows some similarity with that of Tyr/Oct-Dro and Tyr-Loc, there are also clear differences. In particular, phentolamine, chlorpromazine, and mianserine display markedly higher affinities for Lym oa$_1$ than for the insect receptors. As far as the vertebrate adrenergic receptors are concerned, the ligand binding properties of Lym oa$_1$ resemble α_2-adrenergic receptors more than they do α_1- or β-adrenergic receptors. Octopaminergic stimulation of Lym oa$_1$ induces an increase in both inositol phosphates and cAMP ($EC_{50} = 9.1 \times 10^{-7}$ M and 5.1×10^{-6} M, respectively). This is in contrast to the signal transduction pathways described for the related tyramine- and α_2-adrenergic receptors, which couple in an inhibitory way to adenylyl cyclase.

Octopamine, the monohydroxylated analogue of norepinephrine, is a well-established neurotransmitter, neurohormone, and neuromodulator in many invertebrate species (1). In particular, various insect preparations have been used to study the role of octopamine. Because many of the octopamine-mediated responses are connected to adaptation to stressful circumstances, the octopaminergic system has been considered to be the invertebrate equivalent of the vertebrate sympathetic nervous system. Although some early reports describe effects of octopamine in vertebrates (1), it is present in only trace amounts and probably plays no significant role in neurotransmission. This raises the possibility of specifically interfering with octopamine neurotransmission in invertebrates, an issue of considerable interest for the pesticide industry.

Octopamine specifically interacts with octopamine receptors, which belong to the superfamily of G protein-coupled receptors. The pharmacology of octopamine receptors is well documented in several insect species, especially in locusts. Four octopamine receptor subtypes (oct-1, oct-2A, oct-2B, oct-3) have been characterized, each with pharmacological properties (for a review, see Ref. 2). Stimulation of oct-1 receptors induces an increase in intracellular calcium concentration, whereas oct-2A, oct-2B, and oct-3 receptors are positively coupled to AC.

Octopaminergic neurotransmission has been studied not only in insects; biochemical and immunocytochemical studies have demonstrated the presence of octopamine in the brain of several molluscan species (3–7). In addition, the physiology and pharmacology of octopamine receptors have been examined (3, 8–11).

Despite the large interest in the octopaminergic system, the molecular structure of octopamine receptors has not been elucidated. However, two closely related receptors that exhibit highest affinity for tyramine were cloned from *Drosophila* (12, 13) and *Locusta* (14). Tyramine, which is the precur-

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ABBREVIATIONS: AC, adenylyl cyclase; bp, base-pairs; CNS, central nervous system; TM, transmembrane region; HEK, human embryonic kidney; PLC, phospholipase C; IP, inositol phosphate; PCR, polymerase chain reaction; DMEM, Dulbecco's modified Eagle medium.

sor of octopamine, was also suggested to play a role as a neurotransmitter in insects (15), but it is less extensively studied than octopamine. Although tyramine exhibits a higher affinity for the *Drosophila* receptor than does octopamine and tyramine is a more potent inhibitor of AC, both transmitters exhibit similar potencies for raising intracellular calcium levels in cells expressing this receptor (16). Therefore, the *Drosophila* tyramine receptor (13) has been suggested to function as a combined tyramine/octopamine receptor and will be referred to as Tyr/Oct-Dro.

We present the cloning and functional expression of a cDNA encoding a novel G protein-coupled bioamine receptor that is expressed in the CNS of the freshwater snail *Lymnaea stagnalis*. This receptor is designated Lym oa_1 according to the most recent recommendations by the Committee for Receptor Nomenclature of the International Union of Pharmacology (17). The predicted amino acid sequence of Lym oa_1 exhibits highest sequence similarity with the sequence of Tyr/Oct-Dro (12, 13), Tyr-Loc (14), and mammalian α-adrenergic receptors. However, Lym oa_1 clearly differs from these receptors in its high affinity for octopamine and its unique signaling properties: octopamine stimulates both PLC and AC. On the basis of these data, we concluded that we had cloned an octopamine receptor.[2]

Materials and Methods

Animals. Adult *Lymnaea stagnalis* (shell height, 28–34 mm) were bred in the laboratory under standard conditions (18).

Isolation and sequence analysis of the cDNA encoding Lym oa_1. Total RNA (5 μg) was isolated from *L. stagnalis* CNS (19) and reverse-transcribed into cDNA using oligo(dT) primers and Superscript reverse transcriptase (GIBCO BRL, Baltimore, MD). This cDNA was used as a template in a PCR with degenerate oligonucleotides that recognize conserved amino acids in TMs 6 and 7 of G protein-coupled bioamine receptors. The primers and PCR conditions were described previously (20). Sequence analysis (Sequenase, GIBCO BRL) of the PCR products revealed that one of the products showed significant similarity to the mammalian adrenergic receptors. Based on the sequence of this fragment, a specific antisense primer (5′-CAGAAGGATCCGACCTGGTAAATGGTG-3′, based on bp 2563–1584; Fig. 1) was used to isolate the corresponding full-length cDNA clone. A PCR-based library screening was performed on a cDNA library of *L. stagnalis* CNS, cloned in λ-ZAP according to a method described by Gibbons *et al.* (21). The cDNA insert was excised *in vivo* as a pBluescript SK^- phagemid (designated pBS-Lym oa_1) and sequenced on both strands (Sequenase) using a walking primer strategy.

Construction of Lym oa_1 expression vector and expression of Lym oa_1 in mammalian cells. The 5′ part of the open reading frame of Lym oa_1 was amplified in a PCR using pBS-Lym oa_1 as a template and the following two primers: (i) a sense primer (5′-GCA**GGATCC**ACC*ATG*GACTACAAGGACGACGATGACAAGATG-TCACGTGACATCTTCATGA-3′), which starts with a *Bam*HI restriction site (bold letters), followed by a sequence comprising the start codon (italic letters), a region encoding an eight-amino acid FLAG peptide (underlined letters), and a region based on bp 956–977 (see Fig. 1); and (ii) an antisense primer (5′-CAGACTCCTGCCAC-CAAG-3′), based on bp 1394–1411 (see Fig. 1). The resulting PCR product was cloned as a *Bam*HI/*Kpn*I fragment into pBluescript SK^+, and the resulting subclone (pBS-5′Lym oa_1) was sequenced on both strands. The 3′ part of the open reading frame of Lym oa_1 was

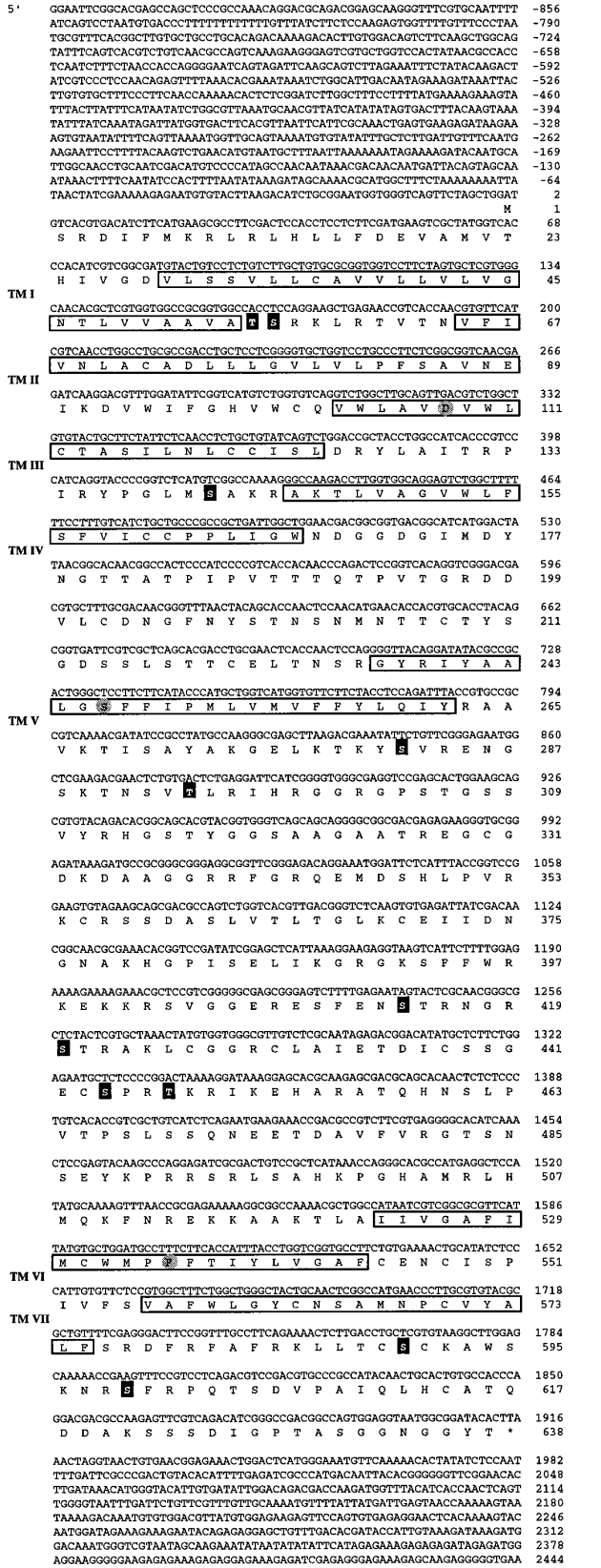

Fig. 1. Nucleotide sequence and predicted amino acid sequence of Lym oa_1. *Boxes*, putative TM regions labeled I–VII; *white letters*, serine or threonine residues represent consensus sites for protein kinase C-mediated phosphorylation; *gray circles*, residues putatively involved in ligand binding.

[2] The sequence of Lym oa_1 has been deposited in the GenBank under accession no. U62771.

amplified similar to the manner described for the 5′ part, using the following primers: (i) a sense primer (5′-TGACCTGCTCGTGTA-AGG-3′), based on bp 2715–2732 (see Fig. 1); and (ii) an antisense primer (5′-GC**TCTAGA**CCTAGT*TTA*AGTGTATCCG-3′), starting with an *Xba*I site (bold letters) and followed by a sequence comprising the predicted stop codon (italic letters), based on bp 2860–2879 (see Fig. 1). A 113-bp *Aat*II/*Xba*I fragment of the resulting PCR product was ligated to a *Kpn*I/*Aat*II fragment of the original cDNA clone, and the resulting subclone (pBS-3′Lym oa_1) was sequenced. The final expression construct (pcDNA3-Lym oa_1) was made by ligation of a *Bam*HI/*Kpn*I fragment of pBS-5′Lym oa_1 and a *Kpn*I/*Xba*I fragment of pBS-3′Lym oa_1 into the *Bam*HI/*Xba*I sites of pcDNA3 (InVitrogen, San Diego, CA). pcDNA3-Lym oa_1 was introduced into HEK 293 cells by calcium phosphate-mediated transfection. Transfected cells were grown in the presence of 0.8 mg/ml geneticin (G-418, GIBCO, Grand Island, NY), and membranes of clonal cell lines were selected for binding to [^{3}H]rauwolscine (5 nM).

Radioligand binding studies. Stably transfected HEK 293 cells were harvested in 50 mM ice-cold Tris·HCl, pH 7.4, and centrifuged for 20 min at 26,000 × *g* at 4°. Cells were lysed by sonication in 5 mM Tris·HCl, pH 7.4, and recentrifuged (20 min), and the membrane pellet was resuspended in 50 mM Tris·HCl, pH 7.4. To determine the dissociation constant (K_D) of [^{3}H]rauwolscine, 20 μg of protein was incubated with increasing concentrations of [^{3}H]rauwolscine (0.1–45 nM) in the presence of 50 mM Tris·HCl, pH 7.4, in a total volume of 250 μl for 30 min at 25°. Yohimbine (5 μM) was added to determine the nonspecific binding. To determine the affinities of several compounds for Lym oa_1, increasing concentrations of inhibitor (10^{-10} to 10^{-4} M) were used to inhibit the binding of 4 nM [^{3}H]rauwolscine. Receptor binding reactions were terminated by filtration over Whatman (Fairfield, NJ) GF/B filters, which were then washed three times with 50 mM ice-cold Tris·HCl, pH 7.4. The radioactivity retained on the filters was measured by liquid scintillation counting. Kaleidagraph 3.0 (Abelbeck/Synergy, Reading, PA) was used to fit the obtained data. Saturation curves were fitted according to the equation: $SB = B_{max} \times [L]/([L] + K_D)$, in which SB is specific binding (total binding minus nonspecific binding), B_{max} is the total number of binding sites, and [L] is the concentration of [^{3}H]rauwolscine. The affinity constant (K_D) was determined from Scatchard analysis plotting specific binding versus specific binding/[L]. Displacement curves were fitted according to the equation: $B = TB - SB \times ([I]/([I] + IC_{50}))^n$, in which B is bound radioactivity, TB is total binding, [I] is the concentration of the inhibitor, and n is the Hill coefficient ($K_i = IC_{50}/[1 + ([L]/K_D)]$)

Chinese hamster ovary cells stably expressing the human α_{2A}-, α_{2B}-, or α_{2C}-adrenergic receptor were used to study the pharmacological profile of the human α-adrenergic receptors. The receptor expression levels of these cell lines, derived from [^{3}H]rauwolscine concentration binding isotherms, were as follows: α_{2A}-adrenergic receptor, B_{max} = 2.8 pmol/mg of protein (K_D = 0.8 nM); α_{2B}-adrenergic receptor, B_{max} = 8.6 pmol/mg of protein (K_D = 1.6 nM); and α_{2C}-adrenergic receptor, B_{max} = 5.1 pmol/mg of protein (K_D = 0.3 nM). For radioligand binding assays, 6–12 μg of membrane protein suspended in 25 mM glycylglycine/NaOH buffer, pH 7.6, was incubated with [^{3}H]rauwolscine (final concentration, 1 nM). Nonspecific binding was determined with oxymetazoline (α_{2A}-adrenergic receptor, 1 μM final concentration) or spiroxatrine (α_{2B}- and α_{2C}-adrenergic receptors, 1 μM final concentration). Assay mixtures were incubated for 30 min at 25° and terminated by rapid filtration over GF/B filters. Filters were washed, and the radioactivity collected on the filters was counted in a liquid scintillation spectrometer. Sigmoidal inhibition curves were calculated by nonlinear regression analysis according to algorithms described by Oestreicher and Pinto (22).

Measurements of IP formation. HEK 293 cells stably expressing Lym oa_1 were grown onto 24-well plates and incubated with 1 μCi of [^{3}H]inositol (Amersham, Arlington Heights, IL)/ml of inositol-free DMEM (GIBCO) supplemented with 10% dialyzed fetal calf serum for 24 hr. Cells were washed once with DMEM and incubated with 10 mM LiCl for 10 min at 37°. Agonists (10^{-3} M to 10^{-9} M) were added and incubated for 60 min at 37°. The medium was removed, and the cells were lysed by sonication in ice-cold chloroform/methanol (1:2). After chloroform extraction, the aqueous phase was incubated with Dowex AG 1X8 anion exchange resin. Resin was washed thoroughly with H_2O, and IPs were eluted with 1 ml of 0.1 M formic acid/1 M ammonium formate. Radiolabeled IPs were measured by liquid scintillation counting.

Measurements of cAMP formation. The cellular concentration of cAMP in HEK 293 cells stably expressing Lym oa_1 was determined as described by Leurs *et al*. (23). Briefly, cells were grown onto 24-well plates and incubated for 20 min at 37° with 300 μM 3-isobutyl-1-methylxanthine and agonists (10^{-3} M to 10^{-9} M) in DMEM. After stimulation of the cells, the medium was aspired, and the cells were lysed by sonication in ice-cold 0.1 N HCl. After neutralization, the extract was incubated with protein kinase A and 30,000 dpm of [2,8-^{3}H]cAMP for 2.5 hr on ice. Reactions were terminated by filtration over a Whatman GF/B filter, and filters were washed three times with 50 mM ice-cold Tris·HCl, pH 7.4. The bound radioactivity was compared with a calibration curve in which 0.25–32 pmol of cAMP was incubated with protein kinase A and [2,8-^{3}H]cAMP.

Results

Cloning of Lym oa_1 cDNA. PCR with degenerated oligonucleotides recognizing conserved regions in TMs 6 and 7 of bioamine receptors resulted in the isolation of several partial receptor cDNA fragments that are expressed in the CNS of the pond snail *L. stagnalis*. One of these fragments showed considerable similarity to the vertebrate α-adrenergic receptors. A full-length cDNA clone (3416 bp) corresponding to this fragment was isolated from a *L. stagnalis* CNS cDNA library (Fig. 1). It consists of a leader sequence of 922 bp, an open reading frame of 1914 bp, and a trailer sequence of 580 bp. The predicted protein (638 amino acids) shows the typical seven hydrophobic regions putatively forming the seven TM domains characteristic for G protein-coupled receptors. An eighth hydrophobic region at the amino-terminal part of the protein, as is present in many invertebrate G protein-coupled neurotransmitter receptors, is not present in Lym oa_1.

A comparison of the predicted amino acid sequence of Lym oa_1 with the Swiss Prot data base revealed highest sequence identity (30–40%) with the *Drosophila* tyramine/octopamine receptor (Tyr/Oct-Dro) (12, 13), the *Locusta* tyramine receptor (Tyr-Loc) (14), and the vertebrate α-adrenergic receptors. When only the TM regions are considered, overall identities are 50% (Tyr/Oct-Dro), 53% (Tyr-Loc), and ~40% (human α_2-adrenergic receptors). A comparison of these amino acid sequences is given in Fig. 2. The second extracellular loop of Lym oa_1 is larger than is usually observed in G protein-coupled neurotransmitter receptors. The amino-terminal region of Lym oa_1 is relatively short and does not contain any consensus sites for *N*-linked glycosylation. These glycosylation sites are a common feature of G protein-coupled receptors and have been associated with membrane trafficking of the receptor. In the intracellular domains of Lym oa_1, 11 consensus sites for phosphorylation by protein kinase C are present that may play a role in receptor desensitization.

Stable expression of Lym oa_1 in HEK 293 cells: pharmacological profile. HEK 293 cells were transfected with pcDNA 3-Lym oa_1 and selected for growth on medium containing 0.8 mg/ml G-418. Several resistant colonies were isolated, and membrane preparations of these clonal cell lines were tested for their ability to bind [^{3}H]rauwolscine.

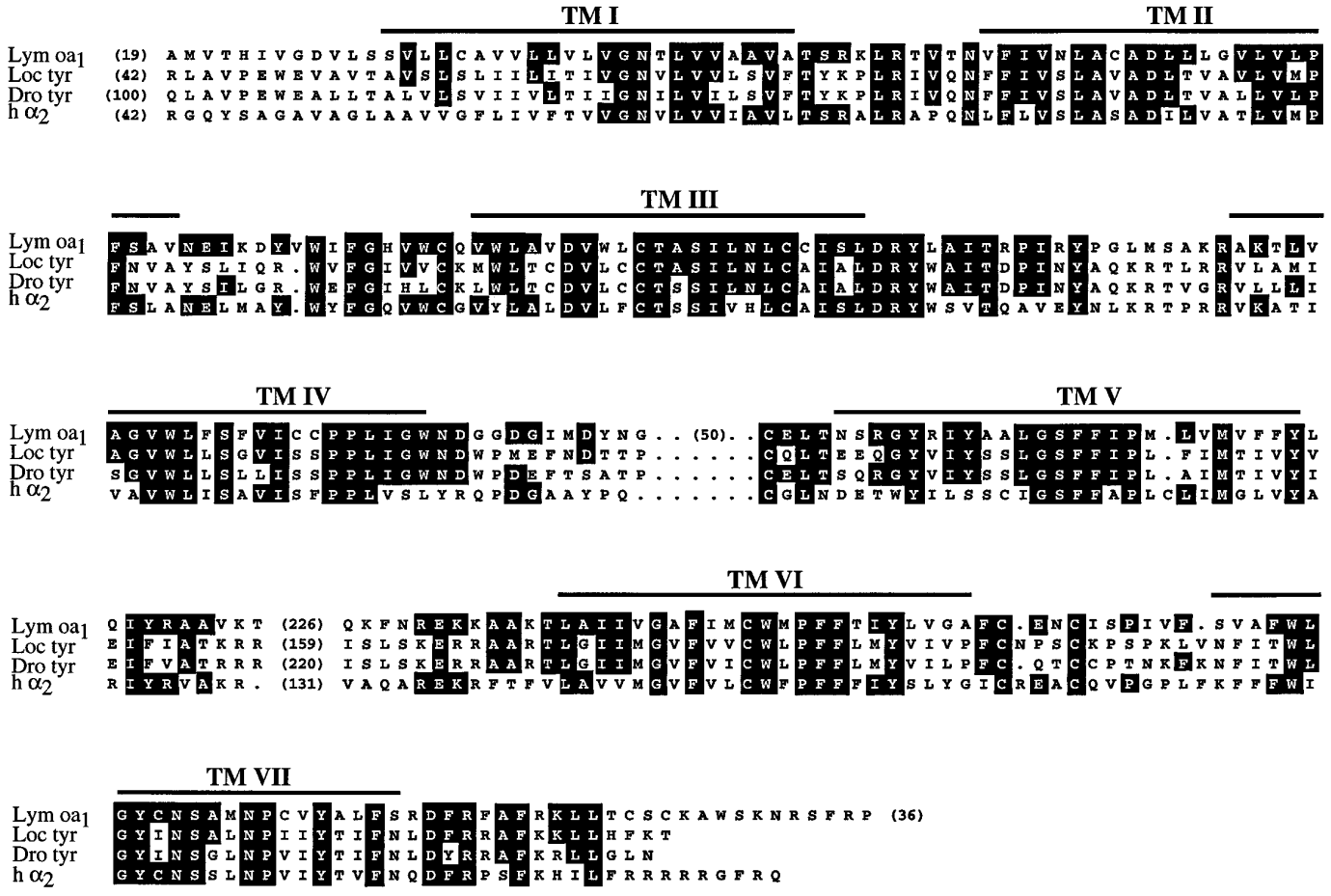

Fig. 2. Alignment of the deduced amino acid sequence of Lym oa_1 with the tyramine receptors cloned from *Drosophila* and *Locusta* (Tyr/Oct-Dro and Tyr-Loc) and with the human platelet α-adrenergic receptor (*h* α_2). *White letters*, identical residues; *overlined*, putative TM regions predicted for Lym oa_1 (labeled I–VII).

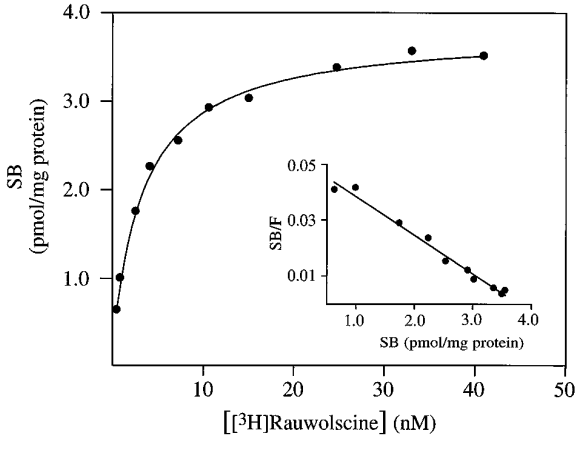

Fig. 3. Saturation binding curve for [^{3}H]rauwolscine binding to the Lym oa_1 receptor expressed in HEK 293 cells. *Inset*, Scatchard plot of the same data; *SB*, specific binding (total binding minus nonspecific binding as determined with 5.10^{-6} M yohimbine binding); *F*, free ligand.

One stable cell line (B_{max} = 3.6 ± 0.2 pmol/mg of protein; five experiments) was selected for further analysis of the pharmacological and signaling properties of Lym oa_1. Fig. 3 shows the saturation binding curve and Scatchard plot for [^{3}H]rauwolscine (K_D = 2.85 nM; five experiments). HEK 293 cells not expressing Lym oa_1 did not show any binding to [^{3}H]rauwolscine (data not shown). To test which naturally occurring agonist could be the ligand for Lym oa_1, several bioamine neurotransmitters were tested for their ability to displace [^{3}H]rauwolscine binding (Table 1). Hill coefficients for all agonist inhibition curves were not significantly different than 1. The rank order of potencies of the tested transmitters was (±)-*p*-octopamine > serotonin = *p*-tyramine > (−)-epinephrine > (−)-norepinephrine > dopamine > histamine. Because of the structural relationship between Lym oa_1 and the adrenergic receptors, we tested several other agonists and antagonists that are known to interact with adrenergic receptors (Table 1). The rank order of potency of those agonists was (±)-*p*-synephrine ≥ clonidine > xylometazoline ≥ phenylephrine ≥ oxymetazoline > B-HT920 >methoxamine, and the rank order of the antagonists was yohimbine > chlorpromazine ≥ spiperone > phentolamine > mianserine ≥ rauwolscine > prazosin > propanolol ≥ alprenolol > pindolol. This profile suggests that Lym oa_1 is more closely related to the α_2- than to the α_1- and β-adrenergic receptors. To determine whether Lym oa_1 exhibits differential homology to one of the α_2-adrenergic receptor subtypes, we performed a similar pharmacological characterization for the human α_{2A}-, α_{2B}-, and α_{2C}-adrenergic receptors (Table 1). No significant correlation could be found between the agonist binding properties of Lym oa_1 and the α_2-adrenergic receptors. For the antagonists, however, Lym oa_1 was more closely correlated to the α_{2B}-adrenergic (r_S = 0.950, $p < 0.001$) than

TABLE 1

pK_i values of various compounds for Lym oa$_1$ and human α_{2A}-, α_{2B}-, and α_{2C}-adrenergic receptors as determined by displacement of [^{3}H]rauwolscine binding

K_i values are mean ± standard deviation, as deduced from at least three independent experiments, each of which was performed in triplicate. In the case of Lym oa$_1$, the concentration of [^{3}H]rauwolscine was 4 nM. In the case of the human α_2-adrenergic receptors, the concentration was 1 nM.

Compound	Lym oa$_1$	Human α_{2A}-adrenergic receptor	Human α_{2B}-adrenergic receptor	Human α_{2C}-adrenergic receptor
			pK_i	
Agonist				
Clonidine	6.57 ± 0.32	7.68 ± 0.26	7.46 ± 0.17	7.00 ± 0.12
(±)-*p*-Synephrine	6.47 ± 0.24	5.30 ± 0.64	4.61 ± 0.28	<5
(±)-*p*-Octopamine	5.72 ± 0.18	4.95 ± 0.42	4.51 ± 0.14	4.84 ± 0.14
Xylomethazoline	5.63 ± 0.03			
Phenylephrine	5.60 ± 0.13	6.09 ± 0.40	4.98 ± 0.49	5.47 ± 0.23
Oxymetazoline	5.56 ± 0.08	8.45 ± 0.25	6.11 ± 0.26	7.26 ± 0.13
B-HT 920	4.80 ± 0.11			
Serotonin	4.43 ± 0.13	5.17 ± 0.08	4.79 ± 0.26	4.94 ± 0.08
p-Tyramine	4.44 ± 0.17	5.62 ± 0.06	5.11 ± 0.00	5.19 ± 0.05
(−)-Epinephrine	4.26 ± 0.08	6.92 ± 0.25	6.11 ± 0.26	7.50 ± 0.55
(−)-Norepinephrine	4.09 ± 0.39	6.67 ± 0.26	6.49 ± 0.11	6.60 ± 0.36
Methoxamine	<4			
Dopamine	<4	5.77 ± 0.36	5.33 ± 0.33	5.76 ± 0.33
Histamine	<4			
Antagonist				
Yohimbine	8.89 ± 0.28	9.62 ± 0.29	8.71 ± 0.10	9.14 ± 0.10
Chlorpromazine	8.53 ± 0.26	6.79 ± 0.57	7.98 ± 0.49	7.14 ± 0.36
Spiperone	8.50 ± 0.31	6.45 ± 0.20	7.64 ± 0.21	7.44 ± 0.36
Phentolamine	8.07 ± 0.14	8.12 ± 0.35	7.78 ± 0.35	7.37 ± 0.29
Mianserine	7.67 ± 0.34	8.15 ± 0.07	7.71 ± 0.19	8.08 ± 0.15
Rauwolscine	7.46 ± 0.02			
Prazosin	7.02 ± 0.05	6.35 ± 0.29	7.54 ± 0.23	7.59 ± 0.19
Alprenolol	5.01 ± 0.41	5.79 ± 0.31	5.34 ± 0.15	5.47 ± 0.15
(−)-Propanolol	4.93 ± 0.15	5.99 ± 0.15	5.21 ± 0.20	5.57 ± 0.06
Pindolol	4.56 ± 0.12	5.79 ± 0.36	4.89 ± 0.04	5.28 ± 0.29

to the α_{2A}-adrenergic ($r_S = 0.820$, $p = 0.007$) or α_{2C}-adrenergic ($r_S = 0.683$, $p < 0.042$) receptor.

Stable expression of Lym oa$_1$ in HEK 293 cells: signal transduction. Activation of octopamine receptors classically results in elevation of the concentration of cAMP in the cell (1). Therefore, we tested the ability of octopamine to stimulate AC in HEK 293 cells stably expressing Lym oa$_1$. Fig. 4 shows the dose-dependent increase in intracellular cAMP levels induced by octopamine and tyramine. Octopamine increases the intracellular cAMP concentration maximally to 9-fold over basal levels, with an EC_{50} value of $5.1 \pm 0.8 \times 10^{-6}$ M. Tyramine could induce only a slight increase in intracellular cAMP levels (2-fold over basal levels) at rather high concentrations ($\geq 10^{-3}$ M). Stimulation of untransfected HEK 293 cells with octopamine or tyramine did not show any elevation in cAMP concentration (data not shown). Related neurotransmitters (epinephrine, norepinephrine, dopamine, serotonin) did not stimulate cAMP formation to a significantly higher degree in HEK 293 cells expressing Lym oa$_1$ than in untransfected cells (data not shown).

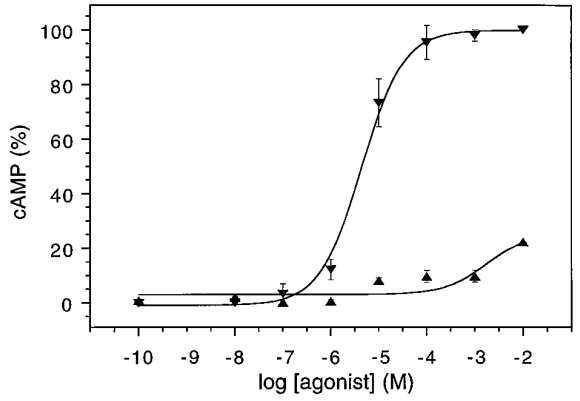

Fig. 4. Concentration-effect relationship of agonist-induced increase in intracellular concentration of cAMP in HEK 293 cells stably transfected with Lym oa$_1$. *Error bars*, standard deviation of the mean value obtained from three experiments, each performed in triplicate. Octopamine (▼) and tyramine (▲) maximally stimulated cAMP levels 9- and 2-fold over basal, respectively. Basal levels were 3 ± 1 pmol/well.

Stimulation of the insect oct-1 receptor induces an increase in intracellular calcium levels (24). In addition, Baines and Downer (25) have shown that the effect of octopamine on phagocytosis in cockroach hemocytes is mediated by inositol trisphosphate. Therefore, we also tested whether octopamine could induce a change in the intracellular concentration of IPs. Indeed, octopamine was able to efficiently increase the amount of IPs to a maximal level of 45-fold over basal (Fig. 5). Also, tyramine, epinephrine, and norepinephrine stimulated IP production, albeit with a lower E_{max} value (±30-fold over basal). EC_{50} values were $9.1 \pm 3.4 \times 10^{-7}$ M for octopamine, $3.0 \pm 1.1 \times 10^{-5}$ M for epinephrine, $5.4 \pm 1.7 \times 10^{-5}$ M for tyramine, and $9.9 \pm 4.0 \times 10^{-5}$ M for norepinephrine. In nontransfected HEK 293 cells, none of the agonists mentioned above was able to induce an increase in IP formation (data not shown).

Discussion

We described the cloning and functional expression of a cDNA that encodes a novel type of G protein-coupled receptor isolated from the CNS of *L. stagnalis*. The predicted protein sequence exhibits highest identity to the tyramine/octopamine receptor from *Drosophila*, the tyramine receptor from

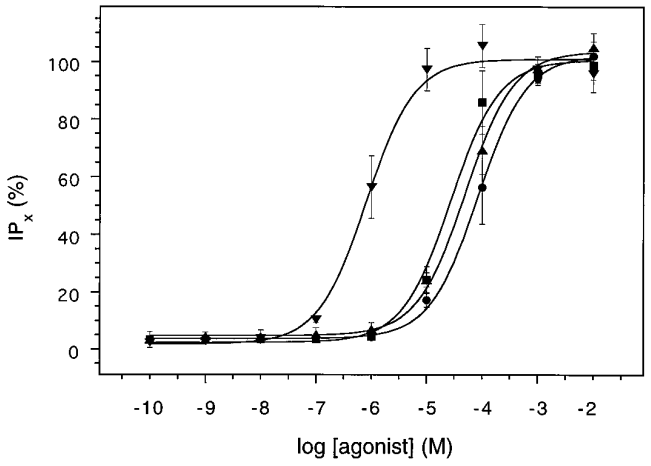

Fig. 5. Concentration-effect relationship of agonist-induced increase in intracellular concentration of IPs in HEK 293 cells stably transfected with Lym oa$_1$. *Error bars*, standard deviation of the mean value obtained from three experiments, each performed in triplicate. Stimulation was performed with octopamine (▼), epinephrine (■), tyramine (▲), and norepinephrine (●). Maximal stimulation was 46-, 33-, 35-, and 31-fold over basal, respectively. Maximal stimulation is plotted as 100% for easier comparison of the concentration dependencies of the different agonist-induced effects on intracellular IP concentration. Basal IP levels were 2.3%, 3.3%, 3.3%, and 3.2%, respectively, and correspond to 292 ± 95 dpm of [^{3}H]IPs/well.

Locusta, and the mammalian α-adrenergic receptors. The extent of this identity (40–50% in TM regions), however, indicates that the *L. stagnalis* receptor is not a species variant of these receptors. Indeed, on permanent expression of the receptor in HEK 293 cells, it shows higher affinity for octopamine than for tyramine or (nor)epinephrine. Furthermore, octopamine is the most potent agonist in stimulation of IP and cAMP formation. These results strongly suggest that we identified an octopamine receptor, and we propose to call it Lym oa$_1$.

The residues that are involved in the interaction between catecholamines and their receptors have been studied extensively. An important source of binding energy between the ligand and the receptor is provided by the ionic interaction with the amine of the ligand and an aspartic acid residue in TM 3. However, both agonists and antagonists interact with this aspartic acid residue. The actual receptor activation is thought to involve an interaction between the catechol moiety of the agonist and residues in TMs 6 and 7. More specifically, the aromatic ring interacts with a phenylalanine residue in TM 6, and the catechol hydroxyl groups form hydrogen bonds with side chains of two serine residues in TM 5. In the β_2-adrenergic receptor, these two serine residues (Ser204 and Ser207) were shown to interact with the *meta*- and *para*-hydroxyl group of the catechol moiety of norepinephrine, respectively (26). In the α_{2A}-adrenergic receptor, Ser204 was shown to be involved in hydrogen bonding with the *para*-hydroxyl group (like its cognate residue in the β_2-adrenergic receptor; 27). In contrast, Ser200 did not seem to participate in receptor agonist interaction. In Lym oa$_1$, the presence of Asp108 in TM 3 and Phe535 in TM 6 is conserved. Interestingly, only one of the two conserved serine residues in TM 5 is present (Ser246). This serine residue corresponds to the serine residues in the α- and β-adrenergic receptors (Ser207 and Ser204, respectively) that are thought to interact with the *para*-hydroxyl group of the catechol moiety of norepinephrine. Because octopamine is a mono-, *para*-hydroxylated catecholamine, the conservation of Ser246 can be considered as indicative for a similar binding of octopamine and norepinephrine to their receptors.

Octopamine is a known neurotransmitter in *L. stagnalis*. Several identified neurons in the CNS have been shown to contain octopamine (7), and recently, [^{3}H]octopamine binding sites in the CNS were pharmacologically characterized.[3] This pharmacological profile, however, differs strongly from the profile of Lym oa$_1$. In particular, the affinities of phentolamine and yohimbine are much higher for Lym oa$_1$ than for the [^{3}H]octopamine binding sites in total *L. stagnalis* CNS. This suggests that in addition to Lym oa$_1$, other octopamine receptor or receptors will be present in *L. stagnalis*.

The pharmacological properties of the oct-1, -2A/-2B, and -3 receptor subtypes from insect tissues have been studied extensively (for a review, see Ref. 2). Interestingly, the ligand binding characteristics of Lym oa$_1$ show no homology with the binding profiles of any of the insect octopamine receptors, suggesting that Lym oa$_1$ is not a species variant of the previously characterized insect octopamine receptor subtypes. Therefore, we suggest that Lym oa$_1$ is the first member of a novel subclass of octopamine receptors.

Because of the structural similarity among octopamine, tyramine, and (nor)epinephrine, we compared the ligand binding properties of Lym oa$_1$ with those of the cloned tyramine and mammalian adrenergic receptors. When the pharmacological profile of Lym oa$_1$ is compared with the profiles of Tyr/Oct-Dro and Tyr-Loc (18–20), there is a better correlation between Lym oa$_1$ and Tyr/Oct-Dro ($r_S > 0.9, p < 0.001$) (12, 13) than between Lym oa$_1$ and Tyr-Loc ($r_S \geq 0.8$, $p = 0.07$) (14). However, phentolamine, chlorpromazine, and mianserine in particular have a markedly higher affinity for Lym oa$_1$ than for the insect receptors. When the pharmacological profile of Lym oa$_1$ is compared with that of the human adrenergic receptors, the affinities of both agonists and antagonists for Lym oa$_1$ indicate that Lym oa$_1$ is more closely related to the α-adrenergic receptors than to the β-adrenergic receptors (illustrated by the high affinity of clonidine and the low affinities of alprenolol, propanolol, and pindolol for Lym oa$_1$). Furthermore, the high affinities of rauwolscine and yohimbine and the moderate affinity of prazosin indicate a closer relationship between Lym oa$_1$ and the α_2-adrenergic receptors than between Lym oa$_1$ and the α_1-adrenergic receptors. Therefore, we wanted to know whether Lym oa$_1$ was most closely related to one of the three α-adrenergic receptor subtypes. Such information could be useful in unraveling the structure-function relationships of α-adrenergic receptors. Concerning the antagonist binding affinities, a better correlation can be found between Lym oa$_1$ and the α_{2B}-adrenergic receptor subtype ($r_S = 0.950, p < 0.001$) than between Lym oa$_1$ and the α_{2A}-adrenergic receptor subtype ($r_S = 0.820, p = 0.007$) and the α_{2C}-adrenergic receptor subtype ($r_S = 0.683, p < 0.042$). However, there is no significant correlation between the agonist binding affinities of Lym oa$_1$ and the α_2-adrenergic receptors. The pharmacological and structural relationships between Lym oa$_1$ and the α_2-adrenergic receptors suggest that the corresponding genes have evolved from a common ancestor. The conservation of the functional prop-

[3] Juhos, S., Z. Hiripi, M. Eckert, J. Rapus, and K. Elekes, personal communication.

erties of octopamine receptors and norepinephrine receptors (e.g., their key role in stress adaptation) is in agreement with such an evolutionary connection.

Most octopamine receptors (i.e., the oct-2 and oct-3 receptor subtypes) have been shown to be positively coupled to AC (1, 2). Activation of the less well-characterized oct-1 receptor subtype has been described to result in an elevation of the intracellular concentrations of both calcium and IPs (24, 25). We have shown that octopamine induces an increase in both IPs and cAMP in cells stably expressing Lym oa_1. We did observe, however, that the coupling of Lym oa_1 to AC seemed to be less efficient than the coupling to PLC. This can be concluded from the fact that the maximal production of cAMP (~20 pmol/well, ~9-fold over basal) is markedly lower than that obtained by stimulation of endogenous ($G_{\alpha s}$ coupled) β-adrenergic receptors in the same HEK 293 cell-line (~48 pmol/well, ~20-fold over basal; data not shown), although the latter receptors are expressed at much lower densities. In contrast, the octopamine-induced stimulation of IP formation is extremely efficient (45-fold over basal). Furthermore, octopamine is the only agonist that can induce a significant increase in cAMP levels in cells expressing Lym oa_1 compared with wild-type HEK 293 cells. Although the stimulation of AC by the activation of endogenous β-adrenergic receptors might mask the effect of (nor)epinephrine on Lym oa_1, this does not hold true for tyramine. On the other hand, all tested agonists elicit a strong IP response to octopamine. Similar situations, in which receptor activation leads to a strong stimulation of phosphatidyl inositol bisphosphate hydrolysis and a weak increase in cAMP levels, have been described for the α_1-adrenergic receptors (28–30), for the m1 and m3 muscarinic acetylcholine receptors (30–36), and for the 5-hydroxytryptamine$_{2A}$ serotonin receptor (37). Such dual signaling can been explained by (i) coupling of the receptor to two different G proteins; (ii) coupling of the receptor to a single G protein that activates PLC, followed by cross-activation of AC by activated protein kinase C or elevated calcium concentrations; or (iii) coupling of the receptor to a single G protein of which the α subunit stimulates AC and the $\beta\gamma$ subunit stimulates PLC. Further experiments are required to find out which of these three possibilities accounts for the dual coupling of Lym oa_1. Furthermore, the presence of secondary signaling pathways can depend on receptor density (28, 38–40) and cell type (29). Therefore, it is worthwhile to investigate whether cell lines expressing lower number of receptors are still capable of dual coupling and whether the coupling is specific for HEK 293 cells.

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