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Octopamine Receptors from the Barnacle *Balanus improvisus* Are Activated by the α_2-Adrenoceptor Agonist Medetomidine[S]

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ABSTRACT

G protein-coupled octopamine receptors of insects and other invertebrates represent counterparts of adrenoceptors in vertebrate animals. The α_2-adrenoceptor agonist medetomidine, which is in clinical use as a veterinary sedative agent, was discovered to inhibit the settling process of barnacles, an important step in the ontogeny of this crustacean species. Settling of barnacles onto ship hulls leads to biofouling that has many harmful practical consequences, and medetomidine is currently under development as a novel type of antifouling agent. We now report that medetomidine induces hyperactivity in the barnacle larvae to disrupt the settling process. To identify the molecular targets of medetomidine, we cloned five octopamine receptors from the barnacle *Balanus improvisus*. We show by phylogenetic analyses that one receptor (BiOctα) belongs to the α-adrenoceptor-like subfamily, and the other four (BiOctβ-R1, BiOctβ-R2, BiOctβ-R3, and BiOctβ-R4) belong to the β-adrenoceptor-like octopamine receptor subfamily. Phylogenetic analyses also indicated that *B. improvisus* has a different repertoire of β-adrenoceptor-like octopamine receptors than insects. When expressed in CHO cells, the cloned receptors were activated by both octopamine and medetomidine, resulting in increased intracellular cAMP or calcium levels. Tyramine activated the receptors but with much lesser potency than octopamine. A hypothesis for receptor discrimination between tyramine and octopamine was generated from a homology three-dimensional model. The characterization of *B. improvisus* octopamine receptors is important for a better functional understanding of these receptors in crustaceans as well as for practical applications in development of environmentally sustainable antifouling agents.

Biofouling of ship hulls has many harmful consequences (e.g., increased fuel consumption and carbon dioxide emissions). Barnacles, a group of species of the subphylum Crustacea, are the most problematic biofouling marine organisms. *Balanus improvisus* is the barnacle species causing the most severe fouling problems in the Baltic Sea, Kattegat, and Skagerrak, whereas *Balanus amphitrite* is more dominant in tropical waters. During the last pelagic stage of the barnacle life cycle (the cyprid), the larvae explore solid surfaces with their antennules to find a suitable site for settling and subsequent metamorphosis into a juvenile sessile barnacle. Antifouling marine paints currently on the market contain toxic heavy metal additives to inhibit cyprid larval settlement, which impose negative consequences on the marine environment. Thus, there is currently a great need to develop new environmentally sustainable antifouling substances.

The α_2-adrenoceptor agonist medetomidine was recently discovered to be capable of efficiently inhibiting the settling process of the barnacles *B. improvisus* and *B. amphitrite* at nonlethal nanomolar concentrations (Dahlström et al., 2000; Dahlström and Elwing, 2006). Panels coated with medetomidine formulated in an acrylate polymer reduced the recruitment of *B. improvisus* to the surface by 96% after 4 weeks

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ABBREVIATIONS: GPCR, G protein-coupled receptor; CHO, Chinese hamster ovary; GTPγS, guanosine 5′-*O*-(3-thio)triphosphate; PCR, polymerase chain reaction; TM, transmembrane helix; RACE, rapid amplification of cDNA ends; 3D, three-dimensional; Oct/Tyr, octopamine/tyramine.

of exposure in Swedish waters (Dahlström et al., 2000). Medetomidine is previously known as a pharmacologically active substance in vertebrates; i.e., it is an α_2-adrenoceptor agonist that is used as a sedative agent in veterinary medicine (Savola et al., 1986; Sinclair, 2003). The mechanism for the inhibitory effect of medetomidine on settling of barnacles is not known, but is assumed to be mediated by G protein-coupled receptors (GPCRs) for octopamine. However, the evidence to support this is so far indirect: 1) medetomidine acts on adrenoceptors in vertebrates, and octopamine serves functions in invertebrates analogous to those of adrenaline and noradrenaline in vertebrates (Roeder, 1999), and 2) clonidine, another α_2-adrenoceptor agonist, has been shown to bind to and activate octopamine receptors in invertebrate species other than barnacles (Gerhardt et al., 1997a,b; Maqueira et al., 2005).

Octopamine receptors of insects and other invertebrates are members of the GPCR superfamily and resemble other biogenic amine receptors, which in addition to octopamine and adrenergic receptors also include receptors for serotonin, dopamine, histamine, and acetylcholine. The octopamine receptors of the fruit fly *Drosophila melanogaster* have been characterized and classified into α-adrenoceptor-like (α-like), β-adrenoceptor-like (β-like), and octopamine/tyramine (Oct/Tyr) receptors, based on their similarity with the vertebrate adrenoceptors. The Oct/Tyr receptors bind both octopamine and tyramine but have a preference for tyramine. One α-like, three β-like, and one Oct/Tyr receptor have been identified in the fruit fly (Evans and Maqueira, 2005). A similar set of octopamine receptor genes has subsequently also been found in other insects for which the whole genome has been sequenced (e.g., *Tribolium castaneum* and *Apis mellifera*) (Hauser et al., 2006, 2008). Relatively little sequence information is available for the octopamine receptor family from invertebrates other than insects, and only a few noninsect receptors have been cloned and functionally characterized. Two putative octopamine receptors from the barnacle *B. amphitrite* have been cloned but not characterized (Isoai et al., 1996; Kawahara et al., 1997); one is an α-like receptor and the other was initially classified as a serotonin receptor but was later shown to be more similar to Oct/Tyr receptors (Evans and Maqueira, 2005).

To learn more about the octopamine receptor family in *B. improvisus* and to better understand the molecular mechanisms of the inhibitory effect of medetomidine on settling, we have cloned and functionally characterized five octopamine receptor family members of this biofouling species. Phylogenetic analysis clearly indicated that four of them belong to the β-like octopamine receptor family, whereas one is of the α-like type. It is noteworthy that some of the members were more highly expressed in the cyprid stage compared with adults. Most importantly from an antifouling perspective, all barnacle octopamine receptors were activated by the antifouling substance medetomidine.

Materials and Methods

For a full description, see the supplementary information.

Rearing of Cyprids and Cyprid Motility Assay. Cypris larvae of *B. improvisus* were produced and reared in a laboratory culture of adult individuals. Cyprid motility was measured by immobilization of the cyprids in agarose gel and monitoring of the number of leg-kicks under a microscope.

Nucleic Acid Preparation and cDNA Synthesis. RNA or genomic DNA was prepared from the barnacles using an RNeasy mini-kit (QIAGEN, Valencia, CA) and the E.Z.N.A Blood DNA Kit (Omega Bio-Tek, Norcross, GA), respectively. RNA was used as template for cDNA synthesis using either the iScript kit (Bio-Rad, Hercules, CA) or the SuperScript III first strand kit (Invitrogen, Carlsbad, CA). The genomic DNA and cDNA were then used for cloning of octopamine receptors and for mRNA expression analysis using real-time PCR.

Cloning of the *B. improvisus* Octopamine Receptors. To clone the octopamine α-like receptor R0, PCR primers corresponding to regions in transmembrane helices I and VII of the known *B. amphitrite* octopamine receptor were used to amplify a fragment of a putative *B. improvisus* homolog. Fragments of the β-like octopamine receptors R1–4 were cloned using two different degenerate primer pairs annealing to conserved regions in biogenic amine receptors. Rapid amplification of cDNA ends (RACE) using the GeneRacer kit (Invitrogen) was used to clone the full-length receptors. The nucleotide sequences for the *B. improvisus* octopamine receptors have been deposited in the GenBank database under the following accession numbers: BiOctα, GU074418; BiOctβ-R1, GU074419; BiOctβ-R2, GU074420; BiOctβ-R3, GU074421; and BiOctβ-R4, GU074422.

Homology Modeling and Phylogenetic Analysis. A homology model of the *B. improvisus* receptors was built based on the turkey β_1-adrenoceptor structure [(Warne et al., 2008); Protein Data Bank identification code 2VT4]. This model was used to identify putative ligand-interacting residues in our barnacle octopamine receptors.

To identify potential orthologs of the cloned *B. improvisus* octopamine receptors, phylogenetic trees were built using the maximum likelihood method. Receptor sequences of various lengths, or binding-pocket residues alone, were used.

Expression of Cloned Receptors in CHO Cells and Second Messenger Assays. To functionally characterize the cloned octopamine receptors, receptor cDNAs were stably transfected into CHO cells. Ligand stimulation of cAMP production was monitored using the LANCE kit (PerkinElmer Life and Analytical Sciences, Waltham, MA). For the α-like receptor R0, ligand stimulation of calcium elevation was measured using the FLIPR Calcium 4 Assay Kit and FlexStation fluorescence plate reader (Molecular Devices, Sunnyvale, CA). Membrane preparations from transfected CHO cells were used to investigate binding of the α_2-adrenoceptor antagonist [^{3}H]RS79948-197 and in the case of R0, also to monitor possible activation of Gα_i-type G proteins by measuring agonist-stimulated binding of [^{35}S]GTPγS.

Real-Time PCR. Real-time PCR was performed to compare the mRNA expression of the *B. improvisus* receptors in cyprids and adults. PCR reactions containing cDNA, receptor specific primers and iQ SYBR Green Supermix (Bio-Rad) were run on an iQ5 iCycler (Bio-Rad).

Results

Medetomidine Activates a Kicking Response in Cyprids. The α_2-adrenoceptor agonist medetomidine is known to result in sedation and locomotor inhibition when given to mammals and fish (Sinclair, 2003; Ruuskanen et al., 2005). Thus, a working hypothesis was that the mode of action of medetomidine on barnacles was also via a sedative action. However, the opposite was found, because medetomidine (10 nM) strongly enhanced kicking of the cyprid larvae, with more than 100 kicks per minute (Fig. 1). Thus, medetomidine elicits different physiological responses in vertebrates and invertebrates (i.e., sedation/locomotor inhibition in vertebrates and hyperactivity in barnacle cyprids). The medetomidine response in barnacles is most likely elicited via octo-

pamine receptors that are functional counterparts of the vertebrate adrenoceptors. That the effect is receptor-mediated is supported by the finding that the α_2-adrenoceptor antagonist atipamezole (100 nM) suppressed the increased kicking activity elicited by medetomidine to near control levels (Fig. 1). To get a better molecular understanding of the physiological response of cyprids to medetomidine, we thus set out to clone and characterize the barnacle octopamine receptor(s).

Cloning of Five Octopamine Receptors from *B. improvisus*. An α-like octopamine receptor from the barnacle *B. amphitrite* was previously cloned by Isoai et al. (1996). To obtain the homologous receptor from *B. improvisus*, PCR-based cloning was carried out using primers corresponding to sequences in transmembrane helices (TMs) I and VII of the *B. amphitrite* receptor and using as templates either genomic DNA or cDNA from a pool of approximately 1000 cyprids. The cypris larvae were derived from a local Swedish west-coast population of adults maintained in a laboratory aquarium setting. Sequence analysis showed that the fragment obtained represented a TM-I to TM-VII region of a *B. improvisus* homolog of the *B. amphitrite* α-like octopamine receptor.

To obtain the full-length receptor cDNA, RACE was performed to identify the sequences of the 5′ and 3′ ends. An open reading frame comprising 1470 nucleotides was obtained from both genomic DNA and cDNA, and this receptor gene was termed R0. Comparison of the nucleotide sequences of the coding regions of six different R0 receptor clones revealed relatively high sequence variability. Pair-wise comparisons of the clones gave a nucleotide diversity of 2 to 3% within the coding region (data not shown). In contrast, the amino acid sequences encoded by the six clones were much more conserved, with only three variable regions (Fig. 2A).

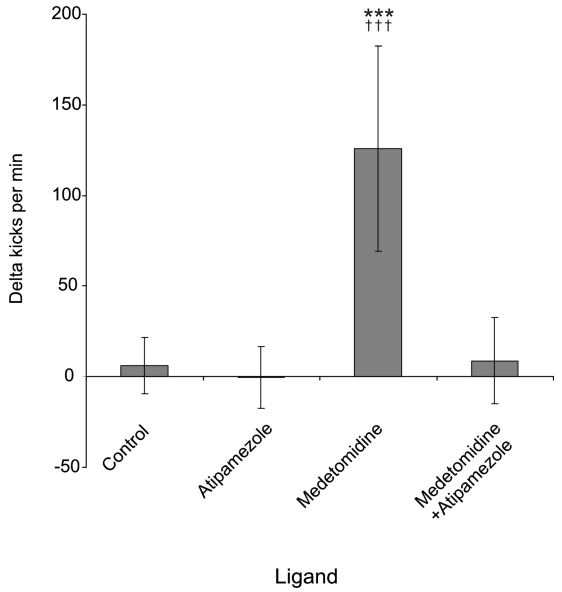

Fig. 1. Medetomidine induces increased leg kicking in cyprids. Cyprids were immobilized in agarose gel and numbers of leg-kicks were recorded before and after the addition of 10 nM medetomidine; 100 nM atipamezole, an α_2-adrenoceptor antagonist; or a combination of these ligands. The difference in the number of kicks before and after ligand addition is shown. The numbers of kicks were significantly greater in the medetomidine-treated cyprids compared with the controls (***, $P < 0.001$) and to the combination of medetomidine and atipamezole (†††, $P < 0.001$) (analysis of variance with Newman-Keuls post hoc test).

The most N-terminal of these regions, located approximately 10 amino acids downstream of the start methionine and consisting of five to nine amino acids, was the most variable. The other two variable regions are located in the long intracellular loop 3 between TM-V and TM-VI.

A comparison of the amino acid sequences of the most frequent variant of the *B. improvisus* R0 clones with the published sequence of the *B. amphitrite* α-like octopamine receptor showed that they are 90% identical at the protein level (Fig. 2B). It is noteworthy that the earlier mentioned variable region in the N terminus of the *B. improvisus* receptor has no counterpart in the shorter N terminus of the *B. amphitrite* receptor. The other 33 differences in amino acids between the two receptors are located outside of the TMs, indicating that the receptors might not be different in ligand binding that is believed to be encompassed mainly within the TM portions of the proteins.

To find more members of the *B. improvisus* octopamine receptor family, in addition to the initially cloned α-like receptor, degenerate PCR primers based on conserved sequences of biogenic amine receptors were used. Two different primer pairs were chosen that had previously enabled cloning of an Oct/Tyr receptor from the grasshopper *Locusta migratoria* and a serotonin receptor from the nematode *Haemonchus contortus* (Smith et al., 2003; Molaei et al., 2005). Twenty-nine clones were isolated using the first primer pair. These clones were sequenced and three different receptor sequences were found. One was identical to the already cloned α-like receptor R0 from *B. improvisus*. A BLAST search versus the NCBI nonredundant protein database showed that the other two variants were most similar to β-like octopamine receptors from various insect species. Of the clones obtained using the other primer pair, 12 were sequenced and also shown to contain three different receptor sequences. A BLAST search showed that one was most similar to dopamine receptors, whereas the other two were most similar to β-like octopamine receptors.

Analogously to the isolation of the full-length α-like R0 receptor, RACE was performed on the obtained fragments of β-like octopamine receptors. Two of the receptors, named R1 and R2, were cloned, yielding open reading frames of 1500 and 1494 base pairs, respectively. Identical results were obtained using either cDNA or genomic DNA as template. The final two receptors clones, named R3 and R4, were cloned from cDNA and had open reading frames of 1071 and 1638 base pairs, respectively. Thus, in total, four different β-like octopamine receptor genes were identified. Amino acid sequence alignment of the four putative β-like octopamine receptors showed that they were quite similar, especially in their TMs (Fig. 3). The clones R1 and R2 exhibited greatest similarity with each other, with 85% identical amino acids in their TMs and an overall identity of 63% for the full-length proteins. It is noteworthy that R1 and R2 have considerably longer intracellular loops 3 compared with both R3 and R4 and also compared with all other known β-like octopamine receptors. Another interesting structural feature is that R4 has an unusually long C terminus compared with other cloned octopamine receptors.

Like in the case of the cloned *B. improvisus* α-like receptor R0, we found nucleotide sequence variation in the range of 2 to 3% between different clones of each individual receptor gene, probably reflecting population heterogeneity (data not

shown). Nucleotide sequence variation in the range 1 to 5% is also seen for a number of other genes (e.g., actin and ribosomal components) identified in a cDNA library constructed from the same cyprid population of *B. improvisus* (M. Alm Rosenblad and A. Blomberg, unpublished data). Great genetic variability within populations has also previously been observed in other marine organisms (e.g., the seasquirt *Ciona savigni*) (Small et al., 2007). The 2 to 3% nucleotide variation between clones of one receptor should be contrasted to the difference between clones representing two different receptors, which is much greater. Of the cloned octopamine receptors, R1 and R2 were most similar to each other having a nucleotide diversity of approximately 23%. We thus regard the variability observed among the individual clones of each of the five octopamine receptors as polymorphism of single genes, although the idea that some of the observed variation relates to the existence of different but very similar receptor genes cannot be totally excluded.

Common GPCR Sequence Features. Analysis of the amino acid sequences of the five cloned *B. improvisus* receptors R0–R4 revealed that they share many common sequence features with other GPCRs. All receptors contained the DRY motif at the end of TM-III, which is well conserved among family A GPCRs and is believed to be involved in receptor activation (Rovati et al., 2007). All receptors contained consensus motifs for phosphorylation by protein kinases A and C that may be important for receptor desensitization (Ferguson et al., 2001). Protein kinase A motifs were found in all receptors in the intracellular loop 3 and/or in the C termini. Consensus motifs for phosphorylation by protein kinase C were found in intracellular loops 2 and 3 of the α-like receptor R0 and in the intracellular loops 3 of R1 and R2. *N*-linked glycosylation sites were found in the N termini of R0, R2, and R3 and in the second extracellular loop of R1. All receptors contained conserved cysteines in extracellular loops 1 and 2, which have been suggested to stabilize receptor structure by forming disulfide bonds (Rader et al., 2004). All receptors also contained cysteines in their proximal C-terminal domains, which are possible targets for palmitoylation.

Search for Orthologs. In the genome of *D. melanogaster*, a repertoire of three β-like octopamine receptors (DmOctβ1, DmOctβ2, and DmOctβ3) has been found, of which all have been cloned and pharmacologically characterized (Maqueira et al., 2005). An alignment of the full-length *B. improvisus* and *D. melanogaster* β-like octopamine receptors showed that all *B. improvisus* receptors were most similar to DmOctβ3, with an identity of 52 to 62% (gaps in the alignments excluded) and a similarity level of 69 to 75% (Table 1). To identify the *D. melanogaster* orthologs of the cloned *B. improvisus* β-like octopamine receptors, and in that way provide evidence for their evolutionary relationships and possible functional roles, a phylogenetic analysis using invertebrate biogenic amine receptors was performed including 1) N- and C-terminally trimmed (to TM-I and TM-VII, data not shown), 2) only highly conserved (mainly membrane spanning regions, data not shown), or 3) full-length sequences (Fig. 4). The R0 clone clustered with the group of α-like octopamine receptors, as expected, whereas R1–R4 clearly clustered with the group of β-like octopamine receptors. However, it was not possible to unambiguously map the *B. improvisus* receptors into the subgroups of the insect β1-, β2-, and β3-like receptors, because they were sometimes found within either of the insect subgroups and sometimes found outside, depending on the phylogenetic analysis

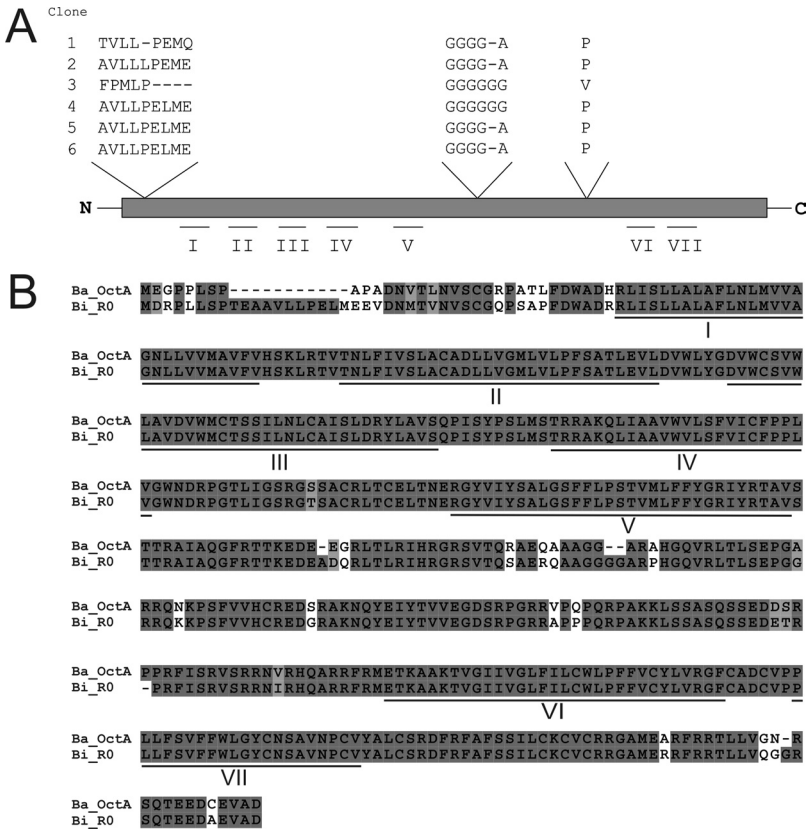

Fig. 2. Variable regions of *B. improvisus* R0 α-like octopamine receptor clones and sequence comparison with the *B. amphitrite* α-like octopamine receptor. A, the amino acid sequences of four genomic DNA and two cDNA R0 clones were aligned. The three regions where the amino acid sequences differ between the clones are indicated. B, the amino acid sequence of the *B. improvisus* R0 receptor and its *B. amphitrite* ortholog were aligned and showed to be 90% identical at the protein level. The *B. amphitrite* receptor has a shorter N terminus than R0 of *B. improvisus*, and there are another 33 amino acid differences, all located outside of the TMs. Identical residues are shaded in dark gray and functionally similar residues are shaded in gray. TMs in the structure of the crystallized β_1-adrenoceptor were used to predict the TMs of R0 by sequence alignment. Putative TMs are underlined.

method and on the sequence regions of the receptors that were used. In addition to the *B. improvisus* receptors identified in this study and the two previously cloned receptors from *B. amphitrite*, the only crustacean for which octopamine receptor sequences are currently available is *Daphnia pulex*. In our phylogenetic studies, one of the putative *D. pulex* octopamine receptors clustered with the β-like receptor group and was usually found on the same branch as the *B. improvisus* R3 receptor.

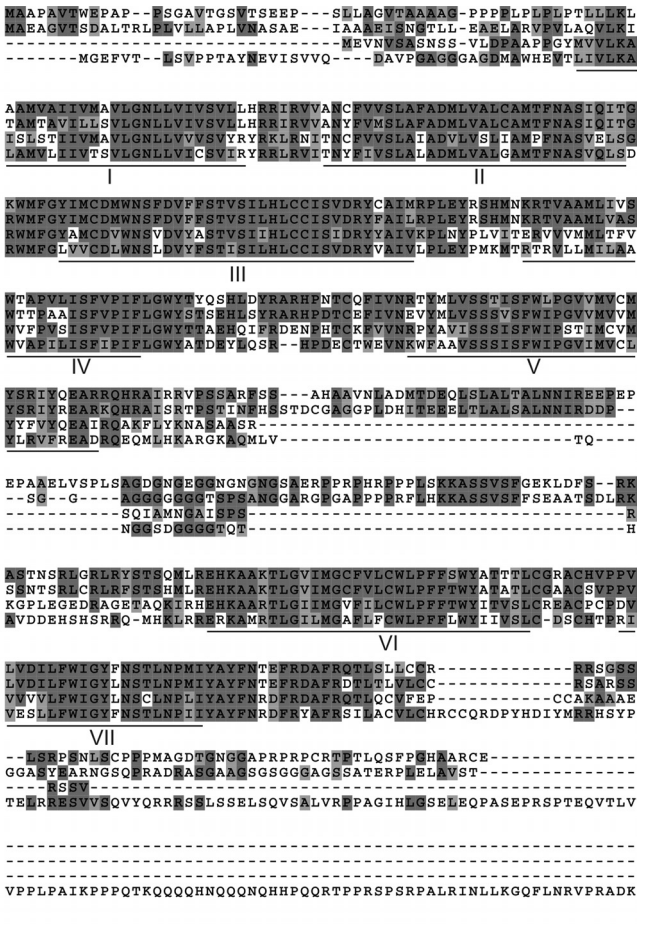

Fig. 3. Amino acid sequence alignment of the *B. improvisus* β-like receptors. The amino acid sequences of the *B. improvisus* β-like octopamine receptors were aligned. Identical residues are shaded in dark gray and functionally similar residues are shaded in gray. TMs in the structure of the crystallized β_1-adrenoceptor were used to predict the TMs of R1–4 by sequence alignment. Putative TMs are underlined.

TABLE 1

Identity and similarity of the cloned β-like octopamine receptors from *B. improvisus* with the β-like octopamine receptors from *D. melanogaster*

The table shows the percentage of identical amino acid residues among all aligned positions [alignment using program ClustalW (http://www.ebi.ac.uk/Tools/clustalw2/index.html)]. The percentage of functionally similar residues is shown in parentheses.

B. improvisus Receptor	*D. melanogaster* β−Adrenoceptor-Like Receptor		
	1	2	3
		%	
R1	45 (61)	43 (61)	52 (70)
R2	44 (62)	41 (60)	52 (69)
R3	53 (71)	54 (69)	62 (75)
R4	46 (65)	49 (68)	55 (72)

Functional Predictions Based on 3D Modeling of the Ligand-Binding Pocket. Classification of receptors is most commonly based on the ligands that activate them or activate a homolog. The conservation of receptor sequences between different species, however, is dependent on many functional and structural determinants, such as ligand binding, interactions with G proteins and other proteins, modification sites, localization signals, etc. Thus, sequence regions related to other functions might perturb a phylogenetic analysis targeting ligand specificity. Therefore, we also classified the receptors based on the putative binding pocket residues alone.

No octopamine receptor protein structure has been experimentally determined. However, related structures of the human β_2-adrenoceptor in complex with the inverse agonist ligand carazolol (Cherezov et al., 2007) and the turkey β_1-adrenoceptor in complex with the antagonist cyanopindolol (Warne et al., 2008) are available. To identify putative ligand-binding pocket residues in our *B. improvisus* octopamine receptors, the turkey β_1-adrenoceptor structure was used as a template to build 3D homology models of the *B. improvisus* β-like octopamine receptors. Several receptor-ligand complex models were then generated by in silico docking of octopamine (Fig. 5A). Many of the receptor-ligand interactions that have been shown to be important for ligand binding in functional studies of biogenic amine receptors are present in the models. These include an aspartate in TM-III (Asp3.32), which is believed to interact with the protonated amine group of the ligand; one or more serines in TM-V that make hydrogen bonds with the hydroxyl groups of the catecholamine ring (Ser5.42, Ser5.43, and Ser5.46); and a cluster of aromatic amino acids in TM-VI that most likely interact with the aromatic ring of the ligand (Trp6.48, Phe6.51, and Phe6.52) (Shi and Javitch, 2002). In one of the models, in which Asn3.29 made a hydrogen bond with the octopamine β-hydroxyl group, 18 residues in the TMs are within 5 Å of the docked ligand and were chosen to represent the binding pocket.

The corresponding 18 positions were extracted from a multiple sequence alignment including our barnacle receptors, the two structurally determined β-adrenoceptors mentioned above, and sequences of selected functionally characterized biogenic amine receptors from humans and various invertebrates. Within the α-like receptor group, all 18 putative ligand-interacting amino acids were completely conserved between the insect octopamine receptors and the *B. improvisus* R0 receptor (Fig. 5B, upper part). The human α-adrenoceptors and the two octopamine receptors from the mollusc *L. stagnalis* differed at 2 to 5 positions compared with the insect and the *B. improvisus* α-like octopamine receptors. The ligand-binding sites of the four *B. improvisus* β-like octopamine receptors R1–R4 were highly similar to the characterized *D. melanogaster* receptors (Fig. 5B, lower part). R3 was identical to the *D. melanogaster* β-like receptors, whereas the ligand binding sites of R1, R2, and R4 differed from those only at one position. A phylogenetic analysis based on the 18 putative ligand-interacting amino acids clearly separated the different classes of biogenic amine receptors into different clades (Fig. S1). As in the phylo-

genetic analyses of the full-length receptors, R1–R4 clustered in this binding pocket analysis with the β-like octopamine receptors, and R0 clustered with the α-like octopamine receptors, supporting their receptor family identity and functional classification.

Functional Studies of the Octopamine Receptors. GPCRs can activate different cellular signaling pathways, leading to modulation of, for example, cAMP and calcium levels. The previously cloned β-like octopamine receptors from *D. melanogaster* and *Aplysia californica* have been shown to mediate stimulation of cAMP formation, whereas most of the cloned α-like octopamine receptors have been reported to signal both via increased cAMP production and increased intracellular calcium concentrations (Gerhardt et al., 1997a; Han et al., 1998; Chang et al., 2000; Grohmann et al., 2003; Bischof and Enan, 2004; Balfanz et al., 2005; Maqueira et al., 2005; Ohtani et al., 2006).

To confirm the phylogenetic analyses indicating that the cloned *B. improvisus* receptors belong to the octopamine receptor family and to investigate whether they are targets for the antifouling substance medetomidine, the five receptors were expressed in stably transfected CHO cells for functional characterization. Ligand-induced changes in cAMP concentrations were analyzed by treating the cells with 1 μM medetomidine, octopamine, tyramine, dopamine or histamine. The β-like receptors R1, R3, and R4 showed relatively strong cAMP responses to octopamine, medetomidine, and tyramine, whereas the responses of cells expressing R2 were weaker but still clearly detectable (Fig. 6A). For the α-like receptor R0, small but significant cAMP increases with medetomidine and octopamine were obtained, but only after coadministration of 100 nM forskolin that activates adenylyl cyclase to a submaximal level (Fig. 6B). No increases in cAMP levels were observed when octopamine, medetomidine, and tyramine were added to nontransfected CHO cells, showing that the obtained responses were mediated by the expressed octopamine receptors (data not shown). Dopamine and histamine activated the receptors only to a small extent or not at all (Fig. 6, A and B). We conclude that the β-like receptors were clearly activated by medetomidine, resulting in increased cellular cAMP production.

To more thoroughly investigate the pharmacology of the

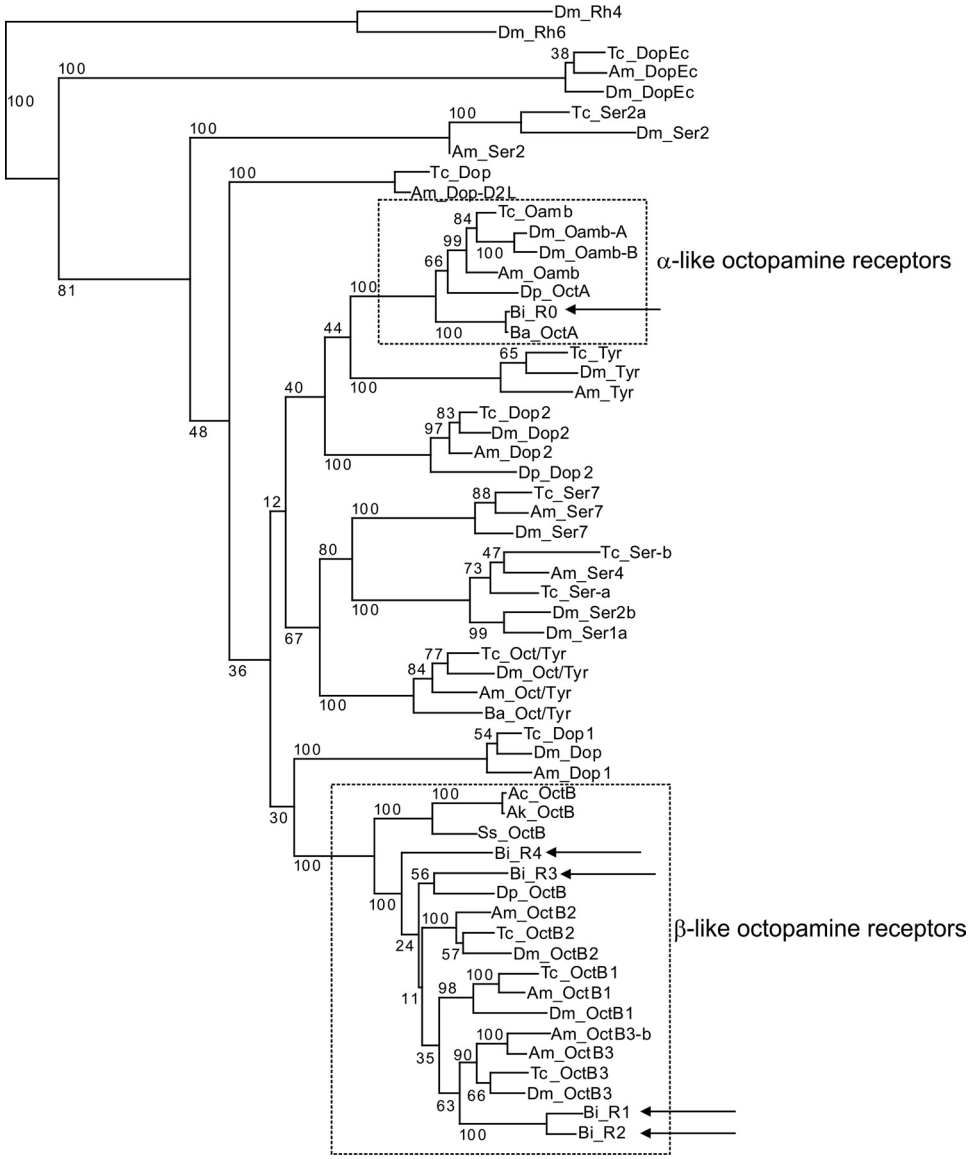

Fig. 4. Phylogenetic tree including *B. improvisus* octopamine receptors and other selected invertebrate full-length biogenic amine receptors. A phylogenetic tree was constructed with PhyML (WAG option) using sequences of the cloned *B. improvisus* (Bi) octopamine receptors, full-length biogenic amine receptors from the three fully sequenced insect species *D. melanogaster* (Dm), the red flour beetle *T. castaneum* (Tc), and the honey bee *A. mellifera* (Am), two octopamine receptors from *B. amphitrite* (Ba), β-like octopamine receptors from the sea hares *A. californica* (Ac) and *Aplysia kurodai* (Ak), the Atlantic surf clam *Spisula sollidisisma* (Ss), as well as three putative octopamine receptors from the waterflea *D. pulex* (Dp). One of the *D. pulex* receptors was in our analysis clearly classified as a dopamine receptor (Dp_Dop2). *D. pulex* labels are chosen according to results from the phylogenetic analyses. *D. melanogaster* Rhodopsin 4 and 6 were used as an outgroup. The clusters of α-like and β-like receptors are indicated with frames. The positions of the *B. improvisus* receptors are indicated with arrows. Bootstrap values are shown at the branches. For accession numbers to the used sequences, see Supplemental Table S4.

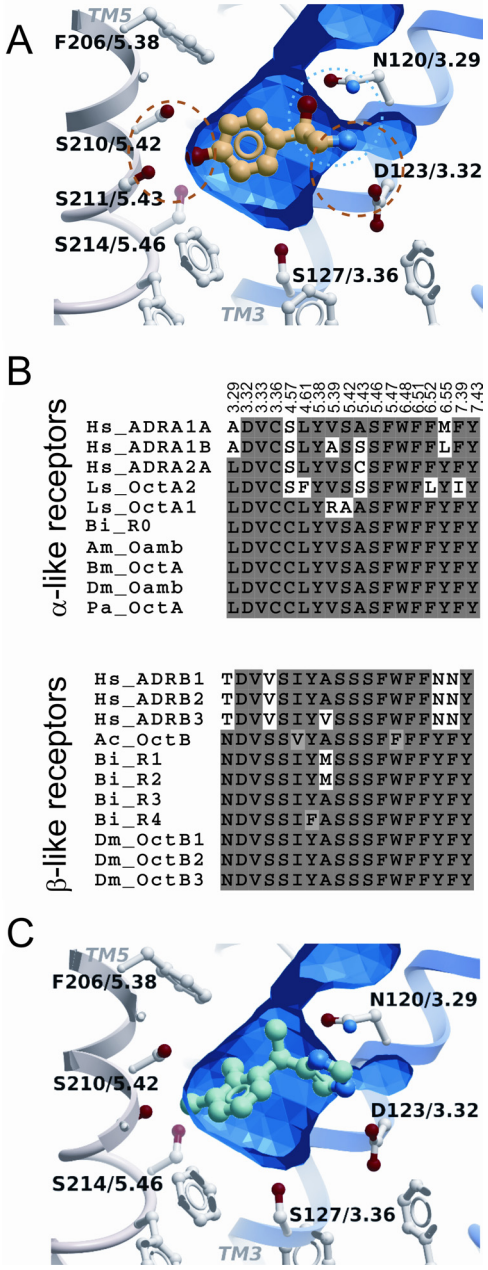

Fig. 5. β-Like octopamine receptor homology model. An octopamine receptor structure model was built using the turkey β_1-adrenoceptor 3D structure as template and putative ligand-interacting residues were identified. A, the model is shown as ribbons and thin ball-and-stick representations. The binding pocket shape is shown as a blue surface, and selected residues and helices are labeled. Residues and pocket surfaces close to the viewer are hidden for clarity, and the extracellular side of the membrane is up. Residues in the TMs are referred to by residue number and the nomenclature of Ballesteros and Weinstein (1995). (*R*)-Octopamine is suggested to bind primarily in two orientations and both include established hydrogen bonding interactions and an ion interaction between the ligand amine and Asp123/3.32 (orange circles). The model shown also includes a hydrogen bond interaction to Asn120/3.29 (blue circle), which may explain the β-like octopamine receptors' binding preference of octopamine over tyramine. The other plausible hydrogen bond interacting residue is also shown (Ser127/3.36). B, by identifying all protein side-chain atoms within 5 Å from octopamine in the R4 receptor homology model, 18 binding site residues were selected. The corresponding amino acids in selected biogenic amine receptors were extracted from a multiple sequence alignment. Putative ligand-interacting residues of the α-like receptors are shown in the upper part and of the β-like receptors in the lower part. C, medetomidine docks in a conformation similar to that of octopamine (A), with electrostatic and hydrogen bonding interactions between the imidazole ring and Asp123/3.32.

β-like octopamine receptors, dose-response curves were generated with the three receptor types that showed the greatest responses to 1 μM concentrations of the investigated ligands (R1, R3, and R4). All three receptors were most sensitive to

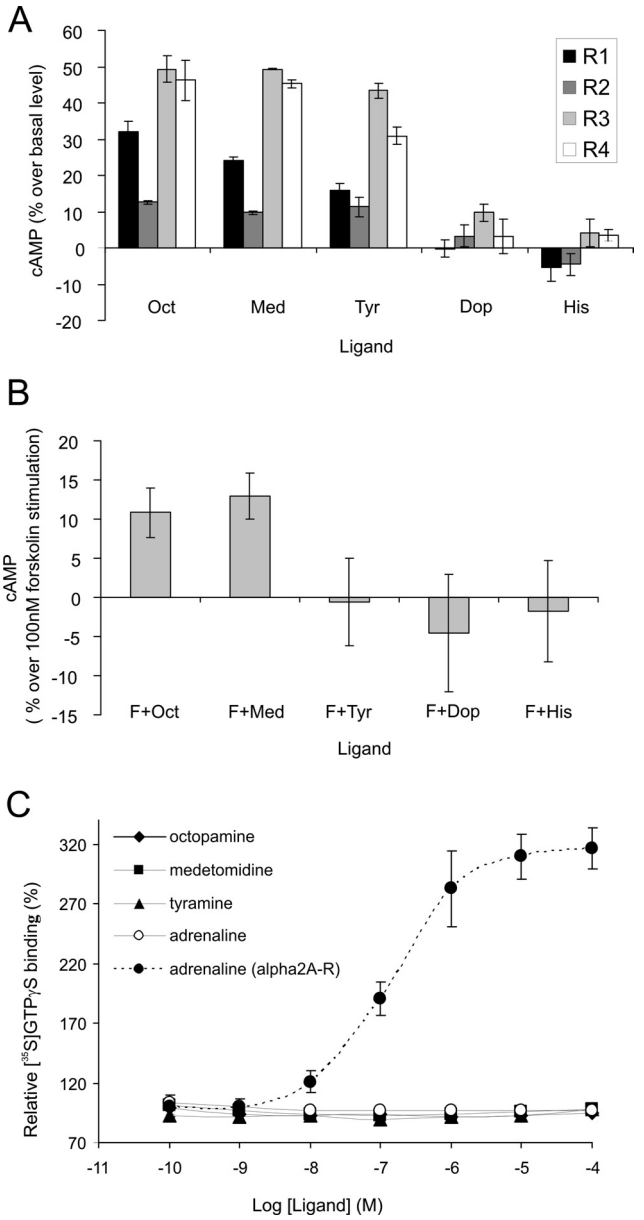

Fig. 6. Ligand-evoked cAMP responses of the cloned octopamine receptors. A, 1 μM octopamine, medetomidine, tyramine, dopamine, or histamine was added to CHO cells expressing the β-like octopamine receptors R1, R2, R3, and R4. The percentage increase in cAMP compared with basal levels is displayed. One experiment with technical duplicates is shown. B, 1 μM octopamine, medetomidine, tyramine, dopamine, or histamine was added together with 100 nM forskolin to CHO cells expressing the α-like receptor R0. The percentage increase over cAMP levels stimulated by 100 nM forskolin alone is displayed. The average of two experiments with six replicates each is shown. In each experiment, the stimulation by octopamine and medetomidine was statistically significant compared with the response induced by forskolin alone (Student's two-tailed *t* test with Bonferroni correction; **, $P < 0.01$). C, agonist-stimulated [^{35}S]GTPγS binding to CHO cell membranes expressing the α-like octopamine receptor R0 was measured. Recombinant human α_{2A}-adrenoceptors that signal via $G\alpha_i$-type G proteins in CHO cells (Peltonen et al., 1998) were used as control to detect adrenaline-stimulated [^{35}S]GTPγS binding and the expected results were obtained (EC_{50} = 140 nM; E_{max} = 210% over basal).

octopamine, with an EC_{50} of approximately 1 nM, followed by medetomidine (EC_{50} approximately 25 times greater than that of octopamine) and tyramine (EC_{50} 57 to 263 times greater than that of octopamine) (Fig. 7; Table 2). The only obvious difference between the three receptors was that R3 seemed to be somewhat more sensitive to tyramine than R1 and R4.

The weak medetomidine-evoked cAMP signal mediated by the α-like receptor R0 was examined further. We first confirmed proper expression of the R0 receptor in the CHO cells with a receptor binding assay using the α_2-adrenoceptor antagonist radioligand [^{3}H]RS79948-197. Membranes prepared from the transfected CHO cells that were used in the functional assay were expressing R0 receptors at a density of approximately 2.4 pmol/mg membrane protein, which is in the range of other recombinant GPCRs expressed in CHO cells. Thus, the weak medetomidine-induced stimulation of cAMP production was apparently not caused by low levels of receptor expression. Next, we monitored agonist-stimulated binding of [^{35}S]GTPγS, a nonhydrolyzable GTP analog often used to investigate activation of G_i-type G proteins (Peltonen et al., 1998). No agonist-stimulated [^{35}S]GTPγS binding was detectable for tyramine, adrenaline, medetomidine, or octopamine in membranes from the R0-transfected CHO cells (Fig. 6C). We concluded that signaling via R0 is not mediated through activation of G_i-type G proteins.

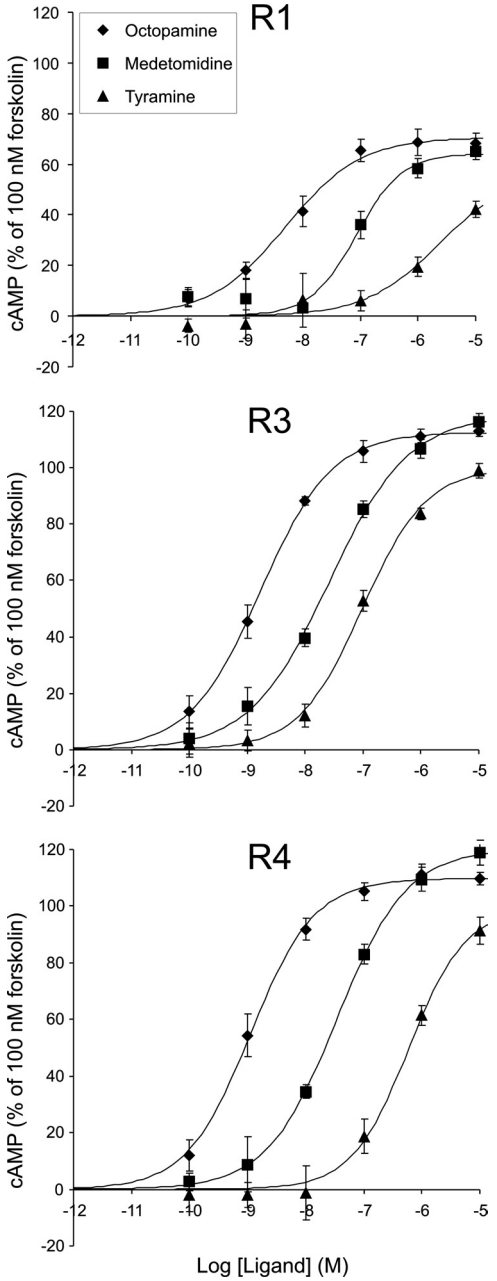

Fig. 7. Dose-response curves for R1, R3, and R4 with octopamine, medetomidine, and tyramine. A wide concentration range (0.1 nM to 10 μM) of the ligands octopamine, medetomidine, and tyramine was added to CHO cells expressing the receptors R1, R3, and R4 (no forskolin was added together with the ligands). The experiment was run three times, two times in duplicate, and one time in triplicate, and the average in percentage of the response obtained with 100 nM forskolin alone is shown. The error bars show the S.E.M.

Because α-like octopamine receptors of other invertebrates have been shown to signal by stimulating intracellular calcium release, in addition to cAMP formation, this possibility was also investigated for R0. Addition of octopamine, medetomidine, and tyramine to R0-expressing CHO cells resulted in concentration-dependent increases in intracellular Ca^{2+} levels (Fig. 8). No Ca^{2+} responses were observed in nontransfected cells (results not shown). Mean ($\pm$ S.E.M.) EC_{50} values of the three tested agonists for the Ca^{2+} responses were 0.35 $\pm$ 0.12 nM for medetomidine, 1.9 $\pm$ 0.4 nM for octopamine, and 160 $\pm$ 30 nM for tyramine (Table 2). On visual inspection of the Ca^{2+} response data (Fig. 8A), it is evident that the responses to medetomidine were more sustained than the responses to octopamine and tyramine. This difference in response kinetics is potentially highly interesting, but its molecular mechanisms were not investigated in this study.

In conclusion, all five cloned *B. improvisus* receptors clearly belong to the octopamine receptor family. All of them are activated by the antifouling substance medetomidine. Our analysis suggests that for the β-like receptors R1–R4, receptor signaling is mediated via stimulation of cAMP formation. For the α-like receptor R0, a small cAMP signal was obtained after activation with octopamine and medetomidine, but only with concomitant forskolin treatment. However, a clear ligand-dependent elevation of calcium levels was detected, indicating that R0 seems to mainly mediate cellular signaling via increasing intracellular calcium concentrations.

TABLE 2

Ligand-dependent second messenger responses of the cloned *B. improvisus* octopamine receptors

Receptors were expressed in CHO cells, and different concentrations of octopamine, medetomidine, and tyramine were added. EC_{50} values of stimulated cAMP formation for the β-like receptors R1, R3, and R4 (n = 3) and of intracellular calcium elevation for the α-like receptor R0 (n = 3–5) are shown. Data are presented as mean $\pm$ S.E.M.

Receptor	EC_{50}		
	Octopamine	Medetomidine	Tyramine
	nM		
Receptor-mediated stimulation of cAMP formation			
R1	4.6 $\pm$ 0.2	99 $\pm$ 15	950 $\pm$ 247
R3	1.6 $\pm$ 0.4	41 $\pm$ 23	91 $\pm$ 33
R4	0.99 $\pm$ 0.22	33 $\pm$ 1	260 $\pm$ 63
Calcium elevation			
R0	1.9 $\pm$ 0.4	0.35 $\pm$ 0.12	160 $\pm$ 30

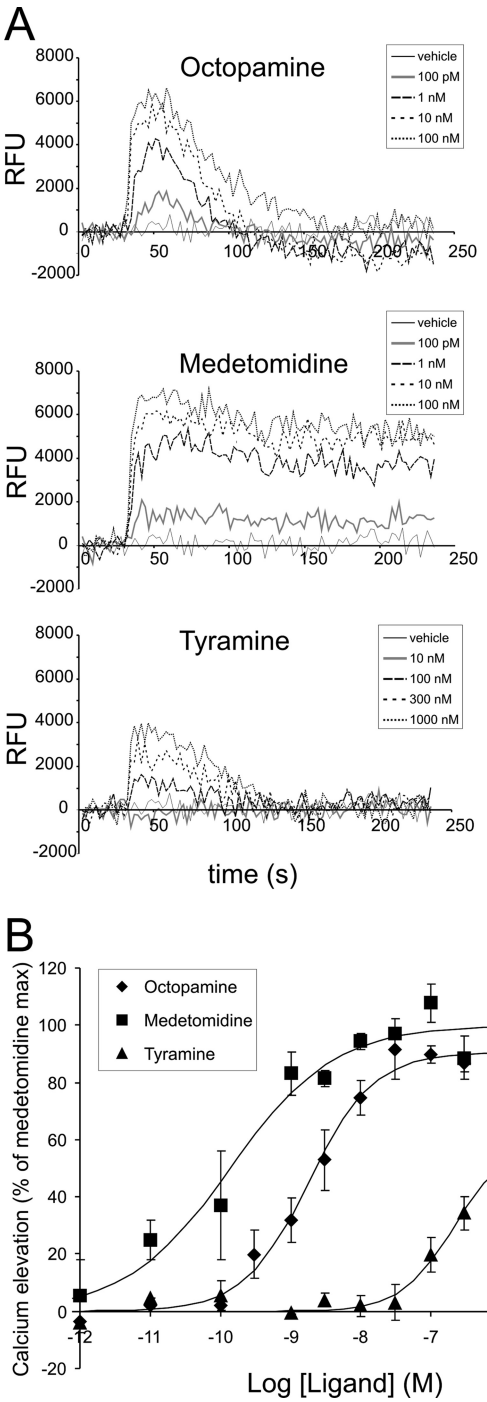

Fig. 8. Ligand-evoked calcium responses of the cloned α-like receptor R0. Different concentrations of octopamine, medetomidine, and tyramine were added to CHO cells expressing the R0 receptor. Increases of intracellular calcium levels were monitored using the FLIPR Calcium 4 Assay Kit (Molecular Devices, Sunnyvale, CA) and the FlexStation automated fluorescence plate reader. A, typical Ca^{2+} response results from individual wells in a single experiment are displayed. Results are shown as relative fluorescence units (RFU) from individual wells exposed to different concentrations of agonists (baseline subtracted). B, concentration-response curves using quadruplicate samples were created. Means ± S.E.M. from three to five experiments are shown after normalization of the Ca^{2+} responses in relation to the maximum response obtained with medetomidine. The maximum responses compared with medetomidine were 83 ± 9% for octopamine and 53 ± 8% for tyramine.

Differential Expression of the *B. improvisus* Octopamine Receptors. The free-swimming cyprid larvae and the permanently attached adults of *B. improvisus* are physiologically very different organisms. Thus, their octopamine receptors might have quite different functional roles. In particular, gene expression in the cyprid stage is of biotechnological interest because this stage responds to medetomidine exposure by not settling. To compare the mRNA expression levels of the cloned *B. improvisus* receptors in cyprids and adults, quantitative real-time PCR was performed on both life stages. It was found that all five octopamine receptor mRNA species were expressed in both cyprids and adults. However, whereas R0, R1, and R2 were approximately equally expressed in both life stages, R3 and R4 were differentially expressed, being significantly more abundant in the cyprid stage (Fig. 9).

Discussion

Cloning and Classification of Barnacle α- and β-like Octopamine Receptors. Octopamine is the invertebrate counterpart of the vertebrate adrenergic transmitters, and it modulates a great variety of invertebrate behaviors. In an attempt to identify the cellular targets in barnacles of the antifouling substance medetomidine, one α-like and four β-like octopamine receptors from *B. improvisus* were cloned and functionally characterized. These are the first functionally characterized crustacean octopamine receptors. The R0 clone is of the α-like type, and we propose that this *B. improvisus* receptor be named BiOctα. Although the four octopamine receptors R1–R4 clearly belong to the β-like octopamine receptor family, they could not unambiguously be mapped into the subgroups of the insect β_1-, β_2-, and β_3-like receptors. *B. improvisus* might thus have a repertoire of β-like octopamine receptors different from that of insects. We therefore refrain from an orthology classification and propose

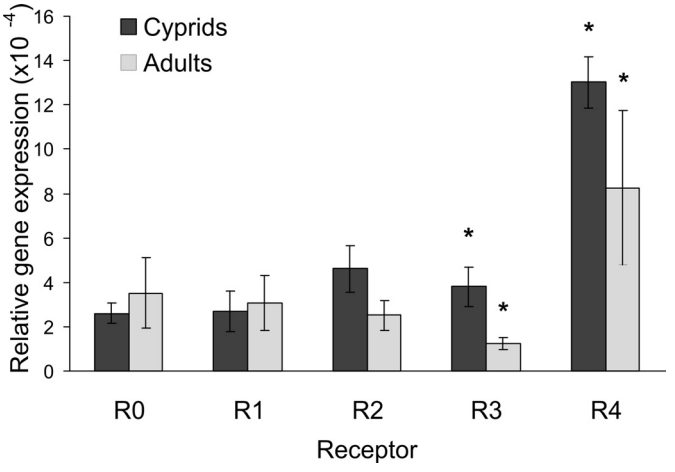

Fig. 9. mRNA expression of the cloned *B. improvisus* receptors. RNA was prepared from six batches of cyprids and from six adult animals, followed by cDNA synthesis.Quantitative PCR was performed with primers for the cloned *B. improvisus* receptors. The average of the relative gene expression ($2^{CT\ (actin)\ -\ CT\ (receptor)}$) from the six samples of cyprids or adults are shown. Error bars display S.E.M. Significant differences between adults and cyprids are indicated (*t* test; *, $p < 0.05$).

to name these cloned receptors BiOctβ-R1 (R1), BiOctβ-R2 (R2), BiOctβ-R3 (R3), and BiOctβ-R4 (R4). The identification of four β-like octopamine receptors is interesting, considering that *D. melanogaster* has only three variants. In this context, it should be noted that *D. melanogaster* achieves greater transcript/protein variability via alternative splicing of at least two of its β-like octopamine receptor genes (Maqueira et al., 2005). The option of alternative splicing to increase the receptor repertoire seems less obvious in *B. improvisus*, because at least BiOctα, BiOctβ-R1, and BiOctβ-R2 are encoded by intronless genes, as shown by cloning of identical receptor sequences from both genomic DNA and cDNA. It will be interesting to further investigate the octopamine receptor repertoire in *B. improvisus* and to compare it with that of other crustaceans as soon as more genome sequences become available.

Binding Selectivity for Octopamine Receptors. The sequence-based octopamine receptor classification was supported by our functional studies because all receptors were activated by octopamine with high efficacy and potency. However, they were also activated by tyramine but with much lower potency. This is in line with both α- and β-like octopamine receptors from *D. melanogaster* (Han et al., 1998; Maqueira et al., 2005). A hypothesis for receptor discrimination between tyramine and octopamine was generated from the homology 3D model of the ligand-binding pocket. Octopamine has a hydroxyl group in the β-position, which corresponds to a hydrogen in tyramine. A potential hydrogen-bonding partner for the β-hydroxyl group of octopamine is Asn3.29, which is conserved in the β-like octopamine receptor family (Fig. 5B). The corresponding residue in the tyramine binding receptors is an aliphatic hydrophobic residue (Ile, Leu, Val), which would agree well with the absence of a β-hydroxyl group in tyramine. The lack of hydrogen bonding between position 3.29 and tyramine might thus explain the lower potency of tyramine compared with octopamine in β-like octopamine receptors.

However, α-like octopamine receptors contain hydrophobic residues at position 3.29, so recognition of the β-hydroxyl group may be different in different receptor types. In support of this, mutational studies of adrenoceptors or octopamine receptors to identify specific ligand interactions have not been conclusive (Chatwin et al., 2003; Ohta et al., 2004; Huang et al., 2007; Perez, 2007). Docking simulations of human α_{2A}-adrenoceptors have indicated that the β-hydroxyl groups in the *R*-enantiomers of noradrenaline-like compounds could form hydrogen bonds with Asp113 (Asp3.32), whereas the *S*-enantiomers of noradrenaline and its analogs cannot form that interaction. Consistent with this, the affinities of the *S*-enantiomers are much weaker than those of the *R*-enantiomers. In this model, the β-hydroxyl group would form a hydrogen bond with one of the two side-chain oxygens of Asp113 (Asp3.32) of α_{2A}-adrenoceptors, whereas the other side-chain oxygen would bind the charged nitrogen of the ligand (Nyrönen et al., 2001). In addition, Huang et al. have suggested Tyr412/6.55 for recognition of the octopamine β-hydroxyl in the α-like octopamine receptor of *Bombyx mori* (Huang et al., 2008). However, this Tyr6.55 residue is at the periphery of the binding pocket in both their model and ours, and its involvement in ligand binding would require a conformational change in the receptor or a water-mediated interaction. Thus, Asp3.32, Asn3.29, and Tyr6.55 could potentially serve similar functions in different octopamine receptors for binding of the β-hydroxyl of octopamine.

Downstream Signaling of the *B. improvisus* Octopamine Receptors. In contrast to the well investigated mammalian G proteins, knowledge about the G protein families of invertebrates is still rather fragmentary. Four barnacle G_α subunits are expressed in the cyprids, and they resemble vertebrate Gα_s-, Gα_q-, Gα_o- and Gα_i-type α-units (M. Alm Rosenblad, S. Falkbring, U. Lind, and A. Blomberg, unpublished data). Gα_s-containing G proteins are activated by a large group of GPCRs and stimulate adenylyl cyclases that convert ATP to cAMP. Gα_q-containing G proteins interact with phospholipase C, leading to a signaling cascade that ends with an increase in cytosolic calcium levels.

Given the clear increase in cAMP production upon ligand activation, Gα_s is most likely to be the G protein isoform that transmits the signal from the *B. improvisus* β-like octopamine receptors. The cAMP response of the α-like receptor BiOctα was weak. However, BiOctα was instead shown to stimulate intracellular calcium elevation, indicating signaling via Gα_q. Previously characterized α-like octopamine receptors have been reported to signal both via increased cAMP production and calcium release (Gerhardt et al., 1997a; Han et al., 1998; Bischof and Enan, 2004; Balfanz et al., 2005; Ohtani et al., 2006). It has been suggested, however, that the main signaling pathway of α-like octopamine receptors is via increases in cytosolic calcium concentrations (Balfanz et al., 2005), which would be in accordance with our results.

Earlier attempts to investigate the effect in vivo of the second messengers cAMP and calcium in cyprid settling have demonstrated the difficulty of conducting such whole-animal experiments, and the results have been inconclusive. Exposure to added second messengers and the use of potent pharmacological inhibitors or activators have in many cases produced opposing results (Clare et al., 1995; Clare, 1996). In addition, such experiments were conducted on whole animals, whereas, if possible, relevant functional organs of the larvae should be investigated, which is technically difficult in these small cyprid larvae.

The Antifouling Substance Medetomidine Activates the *B. improvisus* Octopamine Receptors. We show here that the antifouling substance medetomidine induces a locomotor activation response in barnacle cyprids that is the most likely cause of settling inhibition. Earlier studies have indicated that one of the most important aspects of octopamine signaling in insects is a fight-or-flight response induced by a number of stressful stimuli (e.g., encounters with predators leading to increased muscle performance, increased sensory perception, and increased energy supply) (Roeder, 2005). In addition, octopaminergic insect repellants enhance the motor activity of insects, making them leave the plant (Roeder, 2005).

Our most important finding in the context of biofouling is that octopamine receptors from *B. improvisus* were activated by the antifouling substance medetomidine. In addition, we found that all five octopamine receptor subtypes were expressed in the cyprids and can thus be regarded as potential candidates to mediate the inhibitory effects of medetomidine on the settling process. Dose-response experiments in the cAMP assay with the three β-like octopamine receptors, BiOctβ-R1, BiOctβ-R3, and BiOctβ-R4, showed that the EC_{50} of medetomidine was approximately 30 to 100 nM. The EC_{50}

of medetomidine for R0 in the calcium assay was approximately 0.3 nM. Thus, the in vitro assay results were in rather good agreement with the in vivo EC_{50} estimate previously obtained for cyprid settling inhibition of *B. improvisus*, which is 1 nM (Dahlström and Elwing, 2006). In this perspective, it is worth noting that medetomidine can be docked in a similar orientation as octopamine in our 3D homology model of the octopamine receptors (Fig. 5C). One protonated imidazole-ring nitrogen forms a hydrogen bond-stabilized charge interaction with Asp3.32, and the other nitrogen is in a position to form a hydrogen bond with Asn3.29. The aromatic six-membered ring, however, is tilted by 70° in our model with respect to the octopamine ring to avoid steric clashes between the ring methyl groups of the ligand and residues of the receptor.

It is also of practical interest that all of the cloned octopamine receptors seem to be sensitive to medetomidine. This should make the development of resistance, caused by mutations in the binding sites in each of the octopamine receptors, a less likely event. The somewhat higher sensitivity of BiOctα to medetomidine compared with the β-like receptors suggests that BiOctα might be the main antifouling target. Still, factors in addition to ligand potency determine the functional importance of the different receptor types as antifouling targets. Spatial localization in the cyprids could play a role, and expression in the antennules, which are used by the cyprids to explore surfaces, might be important. The relative expression levels of different receptors might also influence their importance as antifouling targets. Both BiOctβ-R3 and BiOctβ-R4 were found to be more highly expressed in cyprids than in adults, and one could speculate that these two are more important than the other receptors in some cyprid-specific processes such as settling and are thus more likely to be functionally important targets for medetomidine. Our results open up the possibility for population-based analysis of medetomidine sensitivity, for a better understanding of the development of resistance, as well as for developing novel efficacious and environmentally sustainable antifouling substances.

Acknowledgments

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References

Balfanz S, Strünker T, Frings S, and Baumann A (2005) A family of octapamine receptors that specifically induce cyclic AMP production or Ca^{2+} release in *Drosophila melanogaster*. *J Neurochem* **93:**440–451.

Ballesteros JA and Weinstein H (1995) Integrated methods for the construction of three-dimensional models and computational probing of structure-function relations in G protein coupled receptors. *Methods Neurosci* **25:**366–428.

Bischof LJ and Enan EE (2004) Cloning, expression and functional analysis of an octopamine receptor from Periplaneta americana. *Insect Biochem Mol Biol* **34:**511–521.

Chang DJ, Li XC, Lee YS, Kim HK, Kim US, Cho NJ, Lo X, Weiss KR, Kandel ER, and Kaang BK (2000) Activation of a heterologously expressed octopamine receptor coupled only to adenylyl cyclase produces all the features of presynaptic facilitation in aplysia sensory neurons. *Proc Natl Acad Sci USA* **97:**1829–1834.

Chatwin HM, Rudling JE, Patel D, Reale V, and Evans PD (2003) Site-directed mutagenesis studies on the *Drosophila* octopamine/tyramine receptor. *Insect Biochem Mol Biol* **33:**173–184.

Cherezov V, Rosenbaum DM, Hanson MA, Rasmussen SG, Thian FS, Kobilka TS, Choi HJ, Kuhn P, Weis WI, Kobilka BK, et al. (2007) High-resolution crystal structure of an engineered human beta2-adrenergic G protein-coupled receptor. *Science* **318:**1258–1265.

Clare A, Thomas R, and Rittschof D (1995) Evidence for the involvement of cyclic AMP in the pheromonal modulation of barnacle settlement. *J Exp Biol* **198:**655–664.

Clare AS (1996) Signal transduction in barnacle settlement: calcium re-visited. *Biofouling* **10:**141–159.

Dahlström M and Elwing H (2006) Adrenoceptor and other pharmacoactive compounds as putative antifoulants. *Prog Mol Subcell Biol* **42:**171–202.

Dahlström M, Martensson LGE, Jonsson PR, Arnebrant T, and Elwing H (2000) Surface active adrenoceptor compounds prevent the settlement of cyprid larvae of *Balanus improvisus*. *Biofouling* **16:**191–203.

Evans PD and Maqueira B (2005) Insect octopamine receptors: a new classification scheme based on studies of cloned *Drosophila* G-protein coupled receptors. *Invert Neurosci* **5:**111–118.

Ferguson SS (2001) Evolving concepts in G protein-coupled receptor endocytosis: the role in receptor desensitization and signaling. *Pharmacol Rev* **53:**1–24.

Gerhardt CC, Bakker RA, Piek GJ, Planta RJ, Vreugdenhil E, Leysen JE, and Van Heerikhuizen H (1997a) Molecular cloning and pharmacological characterization of a molluscan octopamine receptor. *Mol Pharmacol* **51:**293–300.

Gerhardt CC, Lodder HC, Vincent M, Bakker RA, Planta RJ, Vreugdenhil E, Kits KS, and van Heerikhuizen H (1997b) Cloning and expression of a complementary DNA encoding a molluscan octopamine receptor that couples to chloride channels in HEK293 cells. *J Biol Chem* **272:**6201–6207.

Grohmann L, Blenau W, Erber J, Ebert PR, Strünker T, and Baumann A (2003) Molecular and functional characterization of an octopamine receptor from honeybee (Apis mellifera) brain. *J Neurochem* **86:**725–735.

Han KA, Millar NS, and Davis RL (1998) A novel octopamine receptor with preferential expression in *Drosophila* mushroom bodies. *J Neurosci* **18:**3650–3658.

Hauser F, Cazzamali G, Williamson M, Blenau W, and Grimmelikhuijzen CJ (2006) A review of neurohormone GPCRs present in the fruitfly *Drosophila melanogaster* and the honey bee *Apis mellifera*. *Prog Neurobiol* **80:**1–19.

Hauser F, Cazzamali G, Williamson M, Park Y, Li B, Tanaka Y, Predel R, Neupert S, Schachtner J, Verleyen P, et al. (2008) A genome-wide inventory of neurohormone GPCRs in the red flour beetle *Tribolium castaneum*. *Front Neuroendocrinol* **29:**142–165.

Huang J, Hamasaki T, Ozoe F, Ohta H, Enomoto K, Kataoka H, Sawa Y, Hirota A, and Ozoe Y (2007) Identification of critical structural determinants responsible for octopamine binding to the alpha-adrenergic-like Bombyx mori octopamine receptor. *Biochemistry* **46:**5896–5903.

Huang J, Hamasaki T, Ozoe F, and Ozoe Y (2008) Single amino acid of an octopamine receptor as a molecular switch for distinct G protein couplings. *Biochem Biophys Res Commun* **371:**610–614.

Isoai A, Kawahara H, Okazaki Y, and Shizuri Y (1996) Molecular cloning of a new member of the putative G protein-coupled receptor gene from barnacle *Balanus amphitrite*. *Gene* **175:**95–100.

Kawahara H, Isoai A, and Shizuri Y (1997) Molecular cloning of a putative serotonin receptor gene from barnacle, Balanus amphitrite. *Gene* **184:**245–250.

Maqueira B, Chatwin H, and Evans PD (2005) Identification and characterization of a novel family of *Drosophila* beta-adrenergic-like octopamine G-protein coupled receptors. *J Neurochem* **94:**547–560.

Molaei G, Paluzzi JP, Bendena WG, and Lange AB (2005) Isolation, cloning, and tissue expression of a putative octopamine/tyramine receptor from locust visceral muscle tissues. *Arch Insect Biochem Physiol* **59:**132–149.

Nyrönen T, Pihlavisto M, Peltonen JM, Hoffrén AM, Varis M, Salminen T, Wurster S, Marjamäki A, Kanerva L, Katainen E, et al. (2001) Molecular mechanism for agonist-promoted alpha(2A)-adrenoceptor activation by norepinephrine and epinephrine. *Mol Pharmacol* **59:**1343–1354.

Ohta H, Utsumi T, and Ozoe Y (2004) Amino acid residues involved in interaction with tyramine in the Bombyx mori tyramine receptor. *Insect Mol Biol* **13:**531–538.

Ohtani A, Arai Y, Ozoe F, Ohta H, Narusuye K, Huang J, Enomoto K, Kataoka H, Hirota A, and Ozoe Y (2006) Molecular cloning and heterologous expression of an alpha-adrenergic-like octopamine receptor from the silkworm Bombyx mori. *Insect Mol Biol* **15:**763–772.

Peltonen JM, Pihlavisto M, and Scheinin M (1998) Subtype-specific stimulation of [35S]GTPgammaS binding by recombinant alpha2-adrenoceptors. *Eur J Pharmacol* **355:**275–279.

Perez DM (2007) Structure-function of alpha1-adrenergic receptors. *Biochem Pharmacol* **73:**1051–1062.

Rader AJ, Anderson G, Isin B, Khorana HG, Bahar I, and Klein-Seetharaman J (2004) Identification of core amino acids stabilizing rhodopsin. *Proc Natl Acad Sci USA* **101:**7246–7251.

Roeder T (1999) Octopamine in invertebrates. *Prog Neurobiol* **59:**533–561.

Roeder T (2005) Tyramine and octopamine: ruling behavior and metabolism. *Annu Rev Entomol* **50:**447–477.

Rovati GE, Capra V, and Neubig RR (2007) The highly conserved DRY motif of class A G protein-coupled receptors: beyond the ground state. *Mol Pharmacol* **71:**959–964.

Ruuskanen JO, Peitsaro N, Kaslin JV, Panula P, and Scheinin M (2005) Expression and function of alpha-adrenoceptors in zebrafish: drug effects, mRNA and receptor distributions. *J Neurochem* **94:**1559–1569.

Savola JM, Ruskoaho H, Puurunen J, Salonen JS, and Kärki NT (1986) Evidence for medetomidine as a selective and potent agonist at alpha-2-adrenoceptors. *J Auton Pharmacol* **6:**275–284.

Shi L and Javitch JA (2002) The binding site of aminergic G protein-coupled receptors: the transmembrane segments and second extracellular loop. *Annu Rev Pharmacol Toxicol* **42:**437–467.

Sinclair MD (2003) A review of the physiological effects of alpha2-agonists related

to the clinical use of medetomidine in small animal practice. *Can Vet J* **44:**885–897.
Small KS, Brudno M, Hill MM, and Sidow A (2007) AExtreme genomic variation in a natural population. *Proc Natl Acad Sci USA* **104:**5698–5703.
Smith MW, Borts TL, Emkey R, Cook CA, Wiggins CJ, and Gutierrez JA (2003) Characterization of a novel G-protein coupled receptor from the parasitic nematode *H. contortus* with high affinity for serotonin. *J Neurochem* **86:**255–266.
Warne T, Serrano-Vega MJ, Baker JG, Moukhametzianov R, Edwards PC, Henderson R, Leslie AG, Tate CG, and Schertler GF (2008) Structure of a beta1-adrenergic G-protein-coupled receptor. *Nature* **454:**486–491.

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