Abstract

In invertebrates, the biogenic-amine octopamine is an important physiological regulator. It controls and modulates neuronal development, circadian rhythm, locomotion, ‘fight or flight’ responses, as well as learning and memory. Octopamine mediates its effects by activation of different GTP-binding protein (G protein)-coupled receptor types, which induce either cAMP production or Ca\(^{2+}\) release. Here we describe the functional characterization of two genes from Drosophila melanogaster that encode three octopamine receptors. The first gene (Dmoa1) codes for two polypeptides that are generated by alternative splicing. When heterologously expressed, both receptors cause oscillatory increases of the intracellular Ca\(^{2+}\) concentration in response to applying nanomolar concentrations of octopamine. The second gene (Dmoa2) codes for a receptor that specifically activates adenylate cyclase and causes a rise of intracellular cAMP with an EC\(_{50}\) of \(~3 \times 10^{-8}\) M octopamine. Tyramine, the precursor of octopamine biosynthesis, activates all three receptors at \(\geq 100\)-fold higher concentrations, whereas dopamine and serotonin are non-effective. Developmental expression of Dmoa genes was assessed by RT–PCR. Overlapping but not identical expression patterns were observed for the individual transcripts. The genes characterized in this report encode unique receptors that display signature properties of native octopamine receptors.

Keywords: biogenic amine, Ca\(^{2+}\) oscillation, Ca\(^{2+}\) signalling, G protein-coupled receptor, inositol-1,4,5-trisphosphate, learning and memory.


Octopamine belongs to the group of biogenic amines and plays a major neuromodulatory role in invertebrates (David and Coulon 1985). It has been shown that octopamine modulates neuromuscular transmission in locusts (Malamud et al. 1988), lipid and carbohydrate metabolism under stress (Orchard et al. 1993), and specific forms of learning and behaviour in lobster and honeybee (Livingstone et al. 1980; Burrell and Smith 1995; Menzel and Müller 1996). As octopamine is the monohydroxylated analogue of norepinephrine, it was suggested that the octopaminergic system of invertebrates is the functional correlate of the adrenergic system of vertebrates (for review see Blenau and Baumann 2001). Notably, only trace amounts of octopamine have been detected in vertebrates and its physiological role is still elusive (David and Coulon 1985; Berry 2004).

Numerous pharmacological studies indicate that octopamine acts through activation of specific GTP-binding protein (G protein)-coupled receptors (GPCRs). According to their pharmacological properties and intracellular signalling pathways at least two types of octopamine receptors were defined: OCTOPAMINE 1 (OA1) and OCTOPAMINE 2 (OA2) receptors (Evans and Robb 1993; Roeder et al. 1995; Roeder 1999). Activation of OA1 receptors increases the intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_{i}\)), whereas activation of OA2 receptors stimulates adenylate cyclase and thereby increases the concentration of adenosine 3',5'-cyclic monophosphate ([cAMP])

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Abbreviations used: [Ca\(^{2+}\)]\(_{i}\), intracellular Ca\(^{2+}\) concentration; [cAMP-P]\(_{i}\), intracellular adenosine 3',5'-cyclic monophosphate concentration; Flp-In-293, human embryonic kidney cell line; G418, gentamycin sulfate; G protein, GTP-binding protein; GPCR, G protein-coupled receptor; HEK 293, human embryonic kidney cell line; OA1 receptor, octopamine 1 receptor; OA2 receptor, octopamine 2 receptor; TM, transmembrane.
The physiological role of the octopaminergic system as well as the pharmacological properties of native octopamine receptors were examined in various invertebrate species (for reviews see Roeder 1999; Blenau and Baumann 2001; Roeder et al. 2003). The molecular identification of octopamine receptor genes, however, was lagging behind. During recent years, molecular cloning efforts of biogenic-amine receptors from insects (Arakawa et al. 1990; Saudou et al. 1990; Robb et al. 1994; Von Nickisch-Rosenegk et al. 1996; Han et al. 1998; Grohmann et al. 2003; Bischof and Enan 2004) and molluscs (Gerhardt et al. 1997a,b; Chang et al. 2000) finally led to the isolation of genes that code for octopamine receptors. Some of the polypeptides cause physiological responses in heterologous expression systems similar to those described for native receptors, i.e. cAMP production or Ca\(^{2+}\) release, but two receptors coupled to rather unexpected signalling pathways. A Heliothis receptor (Von Nickisch-Rosenegk et al. 1996) inhibits cAMP production, and one receptor cloned from the pond snail Lymnea (Gerhardt et al. 1997b) activates a Cl\(^{-}\) conductance in the heterologous expression system.

At present, one octopamine/tyramine receptor gene (CG7485: Arakawa et al. 1990; Saudou et al. 1990; Robb et al. 1994) and one octopamine receptor gene have been characterized from Drosophila (OAMB, CG3856: Han et al. 1998). When heterologously expressed, the OAMB receptor causes both Ca\(^{2+}\) release and cAMP production. We have cloned two additional cDNA sequences from Drosophila melanogaster that encode functional octopamine receptors. The Dmoa1 gene gives rise to two receptors that are generated by alternative splicing (DmoA1A, CG3856-PB and DmoA1B, CG3856-PC). The DmoA1A splice-variant is identical to the OAMB receptor (CG3856: Han et al. 1998). The DmoA1A- and DmoA1B receptors share the N-terminal part including transmembrane regions (TM) 1–5, but differ considerably in the C-terminal part. In addition to DmoA1A/1B cDNAs, we cloned a third cDNA (Dmoa2) encoding a different octopamine receptor. HEK 293 and Flp-In-293 (human embryonic kidney cell lines) cells were stably and transiently transfected with Dmoa1A/1B and Dmoa2 constructs to examine the receptors properties. Applying nanomolar concentrations of octopamine to DmoA1A/1B-expressing cells induces oscillations of [Ca\(^{2+}\)], suggesting that both receptors belong to the OA1 receptor subfamily. In contrast to DmoA1 receptors, DmoA2 receptor-activation does not change [Ca\(^{2+}\)], even at octopamine concentrations ≥ 10\(^{-6}\) m. Activation of DmoA2 specifically causes an increase of [cAMP], with an EC\(_{50}\) ≃ 3 × 10\(^{-8}\) m octopamine.

Sequence conservation between DmoA1 and DmoA2 receptors is rather low. Only ~37% of the deduced amino-acid sequences are conserved. In contrast, DmoA1A and DmoA1B receptors share ~67% of similarity. Biogenic-amine receptors that lead to cAMP production have been suggested to be important mediators of learning and memory in invertebrates (Davis 1996; Menzel and Müller 1996; Mayford and Kandel 1999). Therefore, the DmoA2 receptor is a likely candidate for memory acquisition and/or storage functions. In addition, [Ca\(^{2+}\)], oscillations as observed after DmoA1 receptor stimulation might be involved in controlling gene activity and/or fine tuning of neuronal signalling (Dolmetsch et al. 1998; Li et al. 1998). The detailed knowledge of these octopamine receptors will assist to further examine the physiological and functional properties of the octopaminergic system of Drosophila at the molecular level.

Materials and methods

Materials
Radionlabelled compounds, i.e. \(^{32}\)PjDCTP, specific activity >3000 Ci/mmol and the cAMP-assay kit (TRK 432) were from Amersham (Freiburg, Germany). Non-labelled receptor ligands were purchased from RBI and Sigma (Taufkirchen, Germany). Nylon membranes were from Qiagen (Hilden, Germany). The RT–PCR kit (K1402-1) was from BD Biosciences Clontech (Heidelberg, Germany).

Isolation of genomic and cDNA clones from Drosophila libraries
A genomic library of Drosophila was screened under reduced stringency as previously described (Blenau et al. 1998). The probe was a 32P-labelled fragment from Dm\(_{dop1}\) cDNA-clone encoding all transmembrane regions of the Drosophila dopamine D1 receptor (bp 258–1361: Gotzes et al. 1994). Hybridization was performed at 52°C. Restriction fragments of recombinant \(\lambda\)-phages were subcloned into pBluescript SK(–)-vector (pB-vector, Stratagene, Amsterdam, the Netherlands) by standard cloning techniques (Sambrook et al. 1989). Sequencing was performed according to the dideoxy nucleotide sequencing technique (Sanger et al. 1977) using T7 DNA-polymerase (Pharmacia, Freiburg, Germany).

For isolation of full-length cDNA clones, we used PCR-amplification on second strand cDNA and screening of a head-specific library. Two cDNA clones were isolated and completely sequenced on both strands. Clone Dmoa1B contained a long open reading frame encoding a polypeptide of 645 amino acids. The second clone, Dmoa1A, was identical to Dmoa1B in the N-terminal half but lacked the nucleotide sequence encoding the initial 70 amino acid residues. The sequence of the C-terminal half of Dmoa1A was different from that of Dmoa1B, except for some similarity in TM6/7.

The missing 5’-part of Dmoa1A was amplified by PCR using the oligonucleotide 5’-CTCAGGAATTCCACCATGAATGAAACAGAGTGTC-3’ which introduced an EcoRI restriction site and a Kozak consensus-sequence (CCACC: Kozak 1984) immediately 5’ to the initiation codon (ATG). The 3’-primer was 5’-GTCCTCTTCGGAAGAATGTTCAATAAGCAGAG/CAG-3’ where the slash indicates a second primer. The PCR product was cloned into EcoRI-digested pBluescript KI2 (Stratagene, Amsterdam) and sequenced using T7 DNA-polymerase (Pharmacia, Freiburg, Germany).

Isolation of the Dmoa2 gene was achieved after screening the Drosophila genomic database (Adams et al. 2000; NCBI database, BLAST search) with signature sequences derived from molluscan octopamine receptors. A genomic contig (Accession no. AE003739) was identified that displayed high sequence homology to a recently cloned receptor gene from Aplysia (Chang et al. 2000). Based on
this sequence information, specific oligonucleotide primers were synthesized and used for PCR on second strand cDNA preparations from adult heads. The oligonucleotide 5'-ATCATGCTATGGTGTTGTG was used as the 5'-primer, the 3'-primer was 5'-CCGGATCCGGTGTG. The amplified fragment encodes TM4-7 of a biogenic amine receptor. After screening a head-specific cDNA library, several recombinants were obtained. One clone contained the complete open reading frame of the Dmoa2 gene. The sequence data have been submitted to the EBI databases [Accession no. AJ007618, Dmoa1A (CG3856-PB); AJ007617, Dmoa1B (CG3856-PC); AJ617526, Dmoa2 (CG6919)]. To facilitate reading, we will use the abbreviations Dmoa1A (former nomenclature OAMB) and Dmoa1B for the alternatively spliced receptors and Dmoa2 for the second octopamine receptor gene throughout the manuscript.

Polymerase chain reaction on stage-specific cDNA

Tissue from various developmental stages of Drosophila was collected and frozen in liquid nitrogen. RNA was isolated from these tissues by the LiCl/Urea method (Auffray and Rougeon 1980). The amount and purity of RNA was determined photometrically. One microgram of each RNA sample was transcribed into cDNA following the instructions of the RT–PCR kit (K1402-1; BD Biosciences Clontech, Germany). The cDNAs were stored at –80°C.

For PCR-analysis of stage-specific receptor-gene expression, receptor-unique primer pairs were synthesized. Primers were chosen from exons that were separated by one intron at least. For controls, we amplified specific fragments of the Drosophila dopamine D1 receptor (DmDop1) and actin genes. Ten per cent of each cDNA preparation was used for amplification in a final volume of 50 μL as recommended in the kit. Amplification on the corresponding cDNA clones served as a positive control. PCR-conditions were as follows: initial denaturing step for 2 min 30 s at 94°C, followed by 50 cycles with: 30 s at 94°C (denaturing), 30 s at 50°C (annealing), and 40 s at 72°C (extension). PCR products were analyzed by agarose gel electrophoresis.

Construction of pcDmoa expression vectors

For expression in HEK 293 cells, a truncated version of Dmoa1B cDNA containing a Kozak consensus-sequence (Kozak 1984) immediately 5' to the ATG-codon of the open reading frame was constructed by PCR. The oligonucleotide 5'-CTCAAGAAATTTCCA-CCATGATTGAAACAGAGTGC was used as the 5'-primer and the oligonucleotide 5'-CCATCCCTCCGAGCTTGA (bp 1009–1025) was used as the 3'-primer. The PCR product was digested with EcoRI and NaeI. Dmoa1B cDNA in pB-vector was also digested with EcoRI and NaeI. Restriction fragments were gel-purified and ligated into EcoRI cut pcDNA1-amp-vector (Invitrogen, Carlsbad, CA, USA). The PCR-generated part of pcDmoa1B was verified by sequencing.

An expression construct of Dmoa1A was also generated in pcDNA1-vector. As the original cDNA clone (Dmoa1A) lacked the 5'-coding region, this part was substituted for the pcDmoa1B construct. The pcDmoa1B plasmid and Dmoa1A cDNA in pB-vector were cut with EcoRI and BamHI. The 695-bp fragment obtained from pcDmoa1B and the 1762-bp fragment from Dmoa1A, encoding the alternative 3' end, were gel purified and ligated into EcoRI cut pcDNA1-amp-vector. The pcDmoa1A construct was verified by PCR analysis and sequencing. For generation of stably transfected cell lines, both recombinants (pcDmoa1A/1B) were cut with HindIII and Xbal and subcloned into pcDNA1neo-vector (Invitrogen, USA). For transient transfections, Dmoa1A/1B cDNAs were subcloned into HindIII and Xbal cut pcDNA1.amp-vectors (Invitrogen, USA).

Construction of pcDmoa2 was achieved similarly. A unique HindIII restriction site and a Kozak consensus-sequence were introduced immediately 5' to the ATG-codon by PCR. The oligonucleotide 5'-AGGAAAGCTTCCACAGCTACCTGCA- GA was used as the 5'-primer and the oligonucleotide 5'-AGGAATGACGTCGAAGCT (bp 571–589) was used as the 3'-primer. The PCR product was digested with HindIII and AatII. pcDmoa2 cDNA in pB-vector was digested with AatII and EcoRI.

Restriction fragments were gel-purified and ligated into HindIII and EcoRI cut pcDNA1.amp-vector (Invitrogen, USA).

The PCR-generated part of pcDmoa2 was verified by sequencing. A sequence tag encoding the hemagglutinin A (HA) epitope was engineered to the 3'-end of pcDmoa2 by standard cloning techniques. For stable transfection of Flp-In-293 cells, the Dmoa2 encoding fragment was subcloned from pcDNA1.amp- into pcDNA3.1(+) vector (Invitrogen, USA).

Heterologous expression of DmoA receptors

Approximately 10 μg of the Dmoa1A and Dmoa1B recombinants in pcDNA1neo-vector were introduced into exponentially growing (~2 × 105 cells/50 mm dish) HEK 293 cells by a modified calcium phosphate method (Chen and Okayama 1987). Transformed clones were selected in the presence of 1.0 mg/mL of the antibiotic gentamycin sulfate (G418). Isolated foci were propagated and analysed for expression of DmoA1 receptors using Ca2+ fluorescence (see below). The Dmoa2 construct in pcDNA3.1(+) vector was introduced into Flp-In-293 cells and G418-resistant foci were isolated and individually tested for receptor-mediated changes of intracellular second messengers.

Assays to determine cAMP concentrations were performed as described (Grohmann et al. 2003). Mean values of cAMP concentrations were determined in duplicate from two independent experiments at least.

Functional and pharmacological properties of expressed DmoA1 receptors

The ability of DmoA1A, DmoA1B, and DmoA2 to trigger changes in [Ca2+]i, was monitored with the Ca2+-sensitive fluorescence dye Fluo-4. Experiments were done on stably transfected HEK 293 cells. Cells were incubated at 37°C in extracellular solution (in m M: 150 NaCl, 5 KCl, 2 MgCl2,2 CaCl2, 10 HEPES, 30 glucose; pH 7.4) containing 2 μM Fluo-4AM (Molecular Probes, Eugene, OR, USA) and 0.02% Pluronic® F-127 (Sigma, Germany). After 45 min, cells were washed with dye-free extracellular solution. For receptor activation, cells were superfused with extracellular solution containing different concentrations of octopamine or tyramine (see Results). A single cell photon counting system (PhoCal, Life Science Resources, Cambridge, UK) was used to measure [Ca2+]i-dependent changes of Fluo-4 fluorescence. Excitation wavelength was 480 nm (Xe-lamp, 100 W, Nikon). Fluorescence emission was detected at 520–560 nm. The sampling rate of the photon-counting system was adjusted to 100 ms.
Results

Structural features of cloned Drosophila melanogaster octopamine receptors

Homology screening of cDNA libraries led to the isolation of two clones that contained inserts of ~2.2 kb (DmOA1A) and ~2.9 kb (DmOA1B). DmOA1B carries an open reading frame encoding a polypeptide with a calculated molecular mass of 71.7 kDa. The translation initiation-codon (ATG) at positions 460–462 is preceded by nine in-frame stop codons. The open reading frame is terminated by a translational stop-codon (TGA) at positions 2395–2397. The cDNA does not contain a polyadenylation signal but is terminated by six dA residues.

Except for six nucleotide exchanges that do not alter the deduced amino-acid sequence, the Dmoa1A sequence is almost identical to the OAMB gene (Han et al. 1998). The deduced amino-acid sequence of Dmoa1A differs in its C-terminal half from DmOA1B (see Fig. 1) and has a calculated molecular mass of 69.6 kDa. The Dmoa1A cDNA contains a polyadenylation signal (AATAAA) at positions 2422–2427 and is terminated by a stretch of 13 dA residues.

The two polypeptides, DmOA1A and DmOA1B, differ in certain aspects from other members of the family of biogenic-amine receptors. The second extracellular loop between TM4 and TM5 is very long and consists of 147 residues. This loop in GPCRs usually consists of 10–20 residues.
residues (Probst et al. 1992). An interesting feature of the Dmoa1 gene is that it codes for two alternatively spliced transcripts. The gene is composed of four exons coding for identical parts of the DmOA1A/1B receptors and two exons encoding the alternative C-termini. Consensus sequences of splice donor/acceptor sites were found at the exon/intron boundaries. The gene spans ~34.4 kb of genomic DNA (see Fig. 2) and is located at position 92F on the right arm of chromosome 3. Recently, P-element insertions have been described for the Dmoa1 gene which cause impaired oviposition of female flies (Lee et al. 2003).

The Dmoa2 gene was identified by sequence comparison of the Drosophila genome (Adams et al. 2000; NCBI, BLAST search) with the sequence of the Aplysia OA1 gene (ApOA1: Chang et al. 2000). The cloned Drosophila cDNA consists of 1994 bp. The open reading frame encodes a polypeptide of 508 amino-acid residues (DmOA2) with a calculated molecular mass of 56.9 kDa. The open reading frame is terminated by a translational stop-codon at positions 1543–1545. Although DmOA2 shares 66.3% homology with ApOA1, sequence similarity to DmOA1A/1B receptors is rather low. Only 37.2% of the amino-acid residues are conserved between DmOA2 and its DmOA1 relatives. The organization of the Dmoa1 and Dmoa2 genes is quite different (see Fig. 2). Notably, the Dmoa2 gene resembles the Dm dop1 gene, where individual transmembrane regions are encoded by separate exons, too (Gotzes et al. 1994). The Dmoa2 gene is located at position 94B on the right arm of chromosome 3. In contrast to the Dmoa1 gene, no P-element insertions have been reported for the Dmoa2 gene. Screening of the Drosophila genomic database with the Dmoa2 sequence revealed that a related gene (CG6989) is located at position 87C on the same chromosome. The deduced amino-acid sequence of this gene, however, is truncated at both the N- and C-terminus and it is still unknown whether CG6989 encodes a functional receptor protein.

Biogenic amines bind to charged and to hydroxylated amino acids within the binding crevice of the receptor protein (Strader et al. 1995). The DmOA1A/1B and the DmOA2 receptor possess a highly conserved aspartic-acid residue (DmOA1A/B: D106; DmOA2: D187) in TM3 as well as serine residues (DmOA1A/B: S299/303; DmOA2: S276/277/278/280; see Fig. 1) in TM5 that are present in many biogenic-amine receptors (Probst et al. 1992). These residues are likely candidates to bind the neurotransmitter. In addition, invariant hydrophobic residues in TM6 and TM7 of GPCRs are also conserved in DmOA1A/1B (1A: F538/539, W562; 1B: F535/536, W559) and DmOA2 (F366/367, W391) (see Fig. 1). A phylogenetic analysis of invertebrate biogenic-amine receptor sequences revealed that DmOA1 receptors assemble in a clade exclusively containing octopamin receptors. Both the DmOA2 and the ApOA1 receptor are distantly related and form a distinct group in a clade containing receptors binding to tyramine and/or to tyramine and octopamine (Fig. 3).

Stage-specific expression of Dmoa genes
The expression of Dmoa1 and Dmoa2 genes during development of Drosophila was investigated by PCR-amplification on stage-specific cDNA preparations. The cDNA was synthesized on RNA prepared from adult bodies, adult heads, pupae, 3rd instar larvae, and three embryonic stages. The stage-specific expression of Dmoa genes was investigated by PCR-amplification on stage-specific cDNA preparations. The cDNA was synthesized on RNA prepared from adult bodies, adult heads, pupae, 3rd instar larvae, and three embryonic stages. The stage-specific expression of Dmoa genes was investigated by PCR-amplification on stage-specific cDNA preparations. The cDNA was synthesized on RNA prepared from adult bodies, adult heads, pupae, 3rd instar larvae, and three embryonic stages. The stage-specific expression of Dmoa genes was investigated by PCR-amplification on stage-specific cDNA preparations. The cDNA was synthesized on RNA prepared from adult bodies, adult heads, pupae, 3rd instar larvae, and three embryonic stages. The stage-specific expression of Dmoa genes was investigated by PCR-amplification on stage-specific cDNA preparations. The cDNA was synthesized on RNA prepared from adult bodies, adult heads, pupae, 3rd instar larvae, and three embryonic stages.

As a control, we also analyzed the expression profile of Dm dop1. The gene is expressed in adult heads and 3rd instar larvae (Fig. 4a). This result agrees very well with recently published developmental studies of Dm dop1 expression (Hearn et al. 2002; Kim et al. 2003). To monitor the input of cDNA used for PCR-analysis we also performed amplification of the Drosophila actin gene (Fig. 4e).

Functional characterization of DmA1 and DmOA2 receptors
To investigate the intracellular signalling pathways activated by the cloned octopamin receptors, we generated stable cell lines expressing either one of the DmOA isoforms (see
Materials and methods). For each cell line octopamine-dependent changes of $[\text{Ca}^{2+}]_i$ were monitored in single cells by $\text{Ca}^{2+}$ fluorimetry. Figure 5 depicts a series of measure-
ments taken from DmOA1A-expressing cells. The cells did not respond to the application of 1 nM octopamine. Changing the superfusion system to 10 nM octopamine, however, induced repetitive increases of \([\text{Ca}^{2+}]_i\) (Fig. 5a). As shown in Fig. 5(b), \([\text{Ca}^{2+}]_i\) oscillations were stopped when changing the superfusion system from 10 nM octopamine to standard extracellular solution. Superfusion of the same cell with 50 nM octopamine evoked \([\text{Ca}^{2+}]_i\) oscillations with a higher frequency. Raising the octopamine concentration to 1 lM (Fig. 5c) resulted in a single, slowly declining \([\text{Ca}^{2+}]_i\) signal. To test whether the \([\text{Ca}^{2+}]_i\) signals were due to \([\text{Ca}^{2+}]_i\) release from intracellular stores or to \([\text{Ca}^{2+}]_i\) entry from the extracellular side, we performed experiments with nominally no \([\text{Ca}^{2+}]_i\) in the extracellular solution (Ca\(^{2+}\) was substituted for 10 mM EGTA). Application of 1 \(\mu\)M octopamine again resulted in a single \([\text{Ca}^{2+}]_i\) signal (Fig. 5d). From these experiments we conclude that DmOA1A receptors couple to the phospholipase C/inositol triphosphate signalling pathway giving way to \([\text{Ca}^{2+}]_i\) release from intracellular stores.

Octopamine and tyramine share significant structural similarity. Therefore, we tested the potency of tyramine to activate DmOA1A. Figure 5(e) shows the \([\text{Ca}^{2+}]_i\) signals observed after application of different concentrations of tyramine. Low concentrations of tyramine (≤ 100 nM) did not evoke any response. Interestingly, superfusion of DmOA1A-expressing cells with 1 \(\mu\)M tyramine caused \([\text{Ca}^{2+}]_i\) oscillations similar to those observed with 10 nM octopamine. Our observations contrast the results of Han et al. (1998), who described that DmOA1A receptors cause \([\text{Ca}^{2+}]_i\) signals only at high (≥1 \(\mu\)M) octopamine concentrations but cause cAMP production at lower ligand concentrations (see below and Discussion).

In Fig. 6 the data obtained from DmOA1B-expressing cells are shown. As described for the DmOA1A receptor, the DmOA1B receptor also causes \([\text{Ca}^{2+}]_i\) oscillations when activated with octopamine concentrations ≤ 50 nM (Figs 6a and b). Single \([\text{Ca}^{2+}]_i\) signals were observed when superfusing the cells with 1 \(\mu\)M octopamine (Fig. 6c). The DmOA1B-mediated signals are independent from \([\text{Ca}^{2+}]_i\) (Fig. 6d). Oscillations of \([\text{Ca}^{2+}]_i\) were also observed when 1 \(\mu\)M tyramine was superfused onto DmOA1B-expressing cells. However, the cells did not respond to tyramine concentrations ≤ 100 nM (Fig. 6e). Non-transfected cells never showed \([\text{Ca}^{2+}]_i\) signals when superfused with octopamine or tyramine (data not shown).

In contrast to DmOA1A/1B-expressing cell lines, cells expressing the DmOA2 receptor did not show octopamine-dependent \([\text{Ca}^{2+}]_i\) signals (Fig. 7). Application of the Ca\(^{2+}\) ionophore, ionomycin (2 \(\mu\)M), served as a positive control to demonstrate that the Ca\(^{2+}\) detection system worked properly. As DmOA2 and DmOA1 receptors share only 37% sequence homology, we reasoned that DmOA2 might couple to the cAMP signalling pathway. In order to test this hypothesis and to re-examine DmOA1 receptor-evoked cAMP responses, the cAMP production of stably transfected cell lines was measured after incubation with different concentrations of octopamine (Fig. 8a) and tyramine (Fig. 8b). In contrast to non-transfected HEK 293 cells and cell lines expressing either DmOA1 isoform, the DmOA2-expressing cells specifically showed an increase of \([\text{cAMP}]_i\). The response to octopamine was both dose-dependent (EC\(_{50}\) ~3 × 10\(^{-8}\) M) and saturable (Fig. 8a). Compared to octopamine, tyramine was two orders of magnitude less potent (Fig. 8b) in DmOA2-expressing cells.

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Fig. 5 Agonist modulation of \([\text{Ca}^{2+}]_i\) in DmOA1A-expressing cells. Octopamine induced transient increases in \([\text{Ca}^{2+}]_i\). Calcium responses of individual cells expressing the DmOA1A receptor during stimulation with octopamine or tyramine are shown. Cells were loaded with the \([\text{Ca}^{2+}]_i\)-sensitive dye Fluo-4. Relative fluorescence intensity is depicted as counts/100 ms on the ordinate. Application of the ligands is indicated on top of the traces. (a) Superfusion with 1 nM and 10 nM octopamine. (b) Superfusion with 10 nM octopamine. The perfusion was changed for a ligand-free extracellular solution (ES) and subsequently for a test solution containing 50 nM octopamine. (c) Superfusion with 1 nM and 1 \(\mu\)M octopamine. Concentrations (≥ 10 nM) led to oscillations of \([\text{Ca}^{2+}]_i\), whereas 1 \(\mu\)M octopamine caused one single \([\text{Ca}^{2+}]_i\) signal. (d) Superfusion of cells with \([\text{Ca}^{2+}]_i\)-free solutions. Omitting the extracellular \([\text{Ca}^{2+}]_i\) did not change the cellular response to octopamine. (e) Cells were superfused with 50 nM, 100 nM, and 1 \(\mu\)M tyramine. Tyramine evoked \([\text{Ca}^{2+}]_i\) signals only when applied in high (1 \(\mu\)M) concentrations.
In this study we have characterized three members of the octopamine receptor family from *Drosophila melanogaster*. Phylogenetic analysis shows that the receptors belong to two distinct clades of protostomian octopamine receptors. When heterologously expressed, two receptors cause oscillations in \([\text{Ca}^{2+}]_i\) at low concentrations of octopamine. The third receptor specifically leads to an increase of \([\text{cAMP}]_i\) with an EC\(_{50}\) of \(\approx 3 \times 10^{-8} \text{ M}\) octopamine. As native octopamine...
receptors are known to cause either Ca\textsuperscript{2+} signals or to increase [cAMP], the identified Drosophila receptors represent a large fraction of the octopamine receptor gene family in this species.

**Structural properties of DmOA receptors**

Octopamine is an important neuromodulator in protostomians (Monastirioti 1999; Roeder 1999). This biogenic amine binds to GPCRs and leads to specific changes in intracellular second messenger concentrations. Including this study, nine octopamine receptors have been functionally characterized. Three from Drosophila (DmOA1A = OAMB: Han et al. 1998; DmOA1B, DmOA2: this study), one from Apis mellifera (AmOA1: Grohmann et al. 2003), one from Balanus amphitrite (BaOA1: Isoai et al. 1996), one from Periplaneta americana (PaOA1: Bischof and Enan 2004), one from Aplysia californica and Aplysia kurodai (ApOA1: Chang et al. 2000), and two from Lymnea stagnalis (LymOA1, LymOA2: Gerhardt et al. 1997a,b). Additional receptors have been identified that preferentially bind to tyramine and/or to tyramine and octopamine. When phylogenetically analyzed (see Fig. 3), the DmOA1A and DmOA1B sequences assemble in a clade that also contains two insect dopamine receptors (DAMB: Han et al. 1996; AmDOP2: Humphries et al. 2003). Notably, we did not observe activation of DmOA1 receptors by dopamine at all. The DmOA2 receptor, which was identified with the molluscan ApOA1 sequence as bait, coassembles with the ApOA1 receptor in a distinct group within the clade that contains receptors specifically activated by tyramine (Fig. 3). Tyramine is the metabolic precursor of octopamine and only differs from octopamine by the absence of a hydroxyl group on the C\textsubscript{8}-atom. Therefore, one could expect overlapping binding properties of both biogenic amines to the receptor. In fact, we found activation of DmOA2 by tyramine but at ≈100-fold higher concentrations than octopamine. Similar observations have been made for most octopamine receptors studied so far (for review see: Blenau and Baumann 2001).

All three Drosophila receptors share the characteristic seven transmembrane architecture of GPCRs and possess signature amino acid residues implicated in ligand binding (Baldwin et al. 1997; Palczewski et al. 2000; Blenau and Baumann 2001; Filipke et al. 2003). An aspartic-acid residue in TM3 (D100 in DmOA1A/1B; D\textsubscript{187} in DmOA2) can form a salt bridge with the protonated amino group of octopamine. Further stabilization of the ligand in the binding crevice is achieved by hydrogen bonding (Strader et al. 1989, 1995; Woodward et al. 1996) between hydroxyl groups of serine residues in TM5 (S\textsubscript{299/303} in DmOA1A/1B; S\textsubscript{276/277/278/280} in DmOA2) and the hydroxyl group of octopamine’s benzoyl ring.

Activated GPCRs bind to trimeric G proteins to transmit signals to downstream effectors. Interaction between GPCRs and G proteins is mediated by residues in the third intracellular loop and the C-terminus of the receptor (Bourne 1997; Wess 1997). The amino acid sequences of DmOA1A and DmOA1B are very similar at the intracellular juxtamembrane loops flanking TM5, TM6, and TM7. This similarity most likely explains why both splice variants share the signalling properties when expressed in HEK 293 cells. In contrast to DmOA1 receptors, the amino-acid sequence of DmOA2 differs significantly in those domains determining the interaction with G proteins. Consequently, DmOA2 couples to another signalling pathway than DmOA1A/1B.

**Functional coupling to intracellular signalling pathways**

To assess the signalling properties of the cloned Drosophila octopamine receptors, the cDNAs were heterologously expressed in mammalian cell lines. From our previous studies we knew that HEK 293 cells do not express octopamine or tyramine receptors (Gotzes et al. 1994; Blenau et al. 2000; Grohmann et al. 2003). This makes them an ideal testing system compared to various insect cell-lines that express such receptors endogenously (Orr et al. 1992; Hu et al. 1994; Van Poyer et al. 2001; Näsman et al. 2002). Activation of DmOA1A and DmOA1B by octopamine led to Ca\textsuperscript{2+} release from intracellular stores (Fig. 5). At low ligand concentrations (10–100 nM octopamine), we observed Ca\textsuperscript{2+} oscillations. The cellular responses were ligand-specific because tyramine was ~100-fold less efficacious than octopamine. The DmOA1A receptor has been cloned previously and was shown to generate Ca\textsuperscript{2+} signals in transfected cells at octopamine concentrations ≥10\textsuperscript{-5} M (Han et al. 1998). The measurements were done with cell suspensions in a cuvette. The difference in experimental design most likely explains why the oscillatory Ca\textsuperscript{2+} responses that we observed for DmOA1A/1B receptors were overseen before. In contrast to Han et al. (1998), Ca\textsuperscript{2+} fluorimetry of single cells showed that the threshold concentration of octopamine required for DmOA1A/1B-receptor activation was ~10\textsuperscript{-8} M (see Fig. 6). This supports the notion that DmOA1 receptors induce cellular Ca\textsuperscript{2+} signals under physiological relevant octopamine concentrations.

In addition to Ca\textsuperscript{2+} signalling, the DmOA1A (= OAMB) receptor was reported to activate adenylate cyclase (EC\textsubscript{50} 1.9 ± 0.5 × 10\textsuperscript{-7} M; Han et al. 1998). Therefore, we re-examined whether the alternatively spliced DmOA1A and DmOA1B receptors can induce production of cAMP. Compared to DmOA2-mediated responses, only a very small increase in [cAMP] was observed when high octopamine concentrations were applied (see Fig. 8a). In summary, the functional coupling of DmOA1A/1B receptors closely resembled that of AmOA1, a recently characterized orthologous receptor from the honey bee (Grohmann et al. 2003).

It has been shown for several heterologously expressed GPCRs that they can activate different signalling pathways...
when expressed in different cell lines (Robb et al. 1994; Reale et al. 1997). The Dmoa1A cDNA has been previously expressed in Schneider S2 and HEK 293 cells but the cellular responses to receptor activation remained unchanged (Han et al. 1998). Here we have examined the coupling properties of both splice variants of the Dmoa1 gene in HEK 293 cells. Both receptors cause a rise in [Ca^{2+}], a property known from native OCTOPAMINE 1 receptors. In contrast to DmOA1 receptors, the DmOA2 receptor specifically causes a rise in [cAMP]. Half maximal responses were obtained with \( \approx 3 \times 10^{-8} \) M of octopamine. Notably, when high concentrations of octopamine (\( \geq 10^{-6} \) M) were applied to DmOA2-expressing cells, we did not observe any Ca^{2+} signals.

Therefore we classify the DmOA2 receptor as an OCTOPAMINE 2 receptor from Drosophila.

When studying the intracellular signalling pathways activated by the receptors, Ca^{2+} oscillations induced by DmOA1A/1B were an intriguing observation. Ca^{2+} oscillations have been described for many cell types and represent an important form of intracellular signalling (Thomas et al. 1996; Berridge et al. 2000). Notably, two metabotropic glutamate receptors that either cause Ca^{2+} oscillations or induce single Ca^{2+} responses have been described (mGLUR1, mGLUR5: Kawabata et al. 1996, Kawabata et al. 1998). For these receptors it was shown that phosphorylation by PKC in the C-terminus determines whether an oscillatory or a single Ca^{2+} response follows receptor activation. Whether phosphorylation of DmOA1 receptors is necessary to evoke Ca^{2+} oscillations remains to be experimentally tested.

Functional implications of DmOA receptors

Octopamine is an important neuromodulator controlling several physiological functions and behaviours of invertebrate species. Mapping of octopaminergic neurons in Drosophila (Monastirioti et al. 1995) and in the honeybee (Kreissl et al. 1994) revealed that octopaminergic innervation is not restricted to the CNS but is present in the periphery as well. In the insect brain, the mushroom bodies have attracted much attention because they are involved in olfactory learning and memory formation (Erber et al. 1980; Menzel et al. 1994; Meller and Davis 1996; Waddell and Quinn 2001; Heisenberg 2003). A restricted gene expression in this neuropil might therefore indicate that the encoded protein is involved in learning processes. Such examples do exist in Drosophila. Genes participating in cAMP metabolism in the mushroom bodies, i.e. adenylate cyclase (rutabaga: Levin et al. 1992; Zars et al. 2000), phosphodiesterase (dunce: Chen et al. 1986; Dauwalder and Davis 1995), and a G protein (G_{z}: Connolly et al. 1996) severely impair or abolish the learning capability of fruit flies when mutated. It was thus a very interesting result that expression of the Dmoa1A gene was detected in the mushroom bodies of Drosophila (Han et al. 1998). Interestingly though, two recent studies (Lee et al. 2003; Monastirioti 2003) showed that octopamine and the DmOA1A receptor in particular is important for the female reproductive system, where octopamine is required to trigger ovulation. The downstream cellular responses involved in these processes, however, are currently unknown. Based on our results it is reasonable to assume that DmOA1A signalling does not convey onto adenylylate cyclase directly because both splice variants of the gene (DmOA1A/1B) cause Ca^{2+} oscillations rather than cAMP signals. Oscillations of [Ca^{2+}], have been shown to alter gene expression (Dolmetsch et al. 1998; Li et al. 1998). This raises the interesting possibility that DmOA1 receptors control the developmental expression of certain genes in a Ca^{2+}-dependent manner. As DmOA2 causes CAMP production, it would be a good candidate for controlling cAMP levels in the Drosophila CNS. In summary, it is likely to assume that DmOA1 receptors control cellular activity in a Ca^{2+}-dependent fashion, whereas the DmOA2 receptor is well suited to process sensory information and to modulate higher brain function in Drosophila by CAMP signalling.

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