Molecular cloning and characterization of crustacean type-one dopamine receptors: \(D_{1\alpha}^{\text{Pan}}\) and \(D_{1\beta}^{\text{Pan}}\)

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Abstract

Dopamine (DA) differentially modulates identified neurons in the crustacean stomatogastric nervous system (STNS). While the electrophysiological actions of DA have been well characterized, little is known about the dopaminergic transduction cascades operating in this system. As a first step toward illuminating the molecular underpinnings of dopaminergic signal transduction in the crustacean STNS, we have cloned and characterized two type-one DA receptors (DARs) from the spiny lobster (Panulirus interruptus): \(D_{1\alpha}^{\text{Pan}}\) and \(D_{1\beta}^{\text{Pan}}\). We found that the structure and function of these arthropod DARs are well conserved across species. Using a heterologous expression system, we determined that DA, but not serotonin, octopamine, tyramine or histamine activates these receptors. When stably expressed in HEK cells, the \(D_{1\alpha}^{\text{Pan}}\) receptor couples with Gs, and DA elicits an increase in [cAMP]. The \(D_{1\beta}^{\text{Pan}}\) receptor responds to DA with a net increase in [cAMP] that is mediated by Gs and Gz.

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1. Introduction

Monoamines have a variety of physiological and behavioral effects in arthropods (Tierney et al., 2004; Strawn et al., 2000; Cooper and Neckameyer, 1999). The role of neuromodulation in fashioning multiple outputs from a single circuit has long been appreciated. In this regard, important insights have been realized from studies on the STNS in Decapod crustaceans (Hooper and DiCaprio, 2004; Nusbaum and Beenakker, 2002). Peptidergic and monoaminergic modulation of STNS circuits have been studied extensively at the anatomical and electrophysiological levels (Beltz, 1999; Harris-Warrick et al., 1992; Nusbaum, 2002). DA is known to alter both synaptic and intrinsic properties of stomatogastric neurons in a cell specific manner (Bucher et al., 2003; Cleland and Selverston, 1997; Harris-Warrick et al., 1998; Johnson et al., 2003a; Kloppenburg et al., 1999; Peck et al., 2001); however, little is known about the signal transduction cascades that generate these physiological responses.

Dopaminergic responses are mediated through multiple DARs that comprise an evolutionarily conserved family of G protein coupled receptors (GPCRs). DARs are thought to have evolved initially from gene duplication and drift leading to 2 related paralogous genes defining two different subfamilies: \(D_1\) and \(D_2\) (Callier et al., 2003; Kapsimali et al., 2003; Le Crom et al., 2003). To date, all DARs can be broadly classified into these two subfamilies on the basis of conserved structure and signaling mechanisms. In general, type 1 DARs preferentially couple to Gs to increase adenylyl cyclase activity while type 2 receptors preferentially couple with G\(_i\)/G\(_o\) to decrease adenylyl cyclase activity (Neve et al., 2004). Pharmacology is also used to classify vertebrate DARs. Pharmacological profiles are not conserved across vertebrate/invertebrate lines, however, so vertebrate pharmacology cannot be used when classifying arthropod receptors as \(D_1\) vs. \(D_2\).

The natural history of \(D_1\) receptors has been well studied for vertebrates, but much less is known for vertebrate \(D_2\) receptors. In addition, the orthologous relationships for vertebrate and invertebrate DARs are unknown (Kapsimali et al., 2003). Seven DAR subtypes exist in the phylum chordata: four \(D_1\) subtypes (\(D_{1A}, D_{1B}, D_{1C}, D_{1D}\)) and three \(D_2\) subtypes (\(D_2, D_3, D_4\)). A given class (e.g., mammal, teleost, reptile, etc.) may possess
only a subset of the seven. For example, only five DAR subtypes are represented in mammals: D1/D1A, D1B/D5, D2, D3, D4. There are three well-characterized DARs in the phylum arthropoda (Blenau et al., 1998; Feng et al., 1996; Gotzes et al., 1994; Han et al., 1996; Hearn et al., 2002; Sugamori et al., 1995). Two of these receptors can be classified as type 1, and one of these receptors can be classified as type 2. A fourth arthropod receptor that responds to DA with a slight, but significant increase in cAMP has recently been cloned (Srivastava et al., 2005). This receptor also responds strongly to ecdy steroids, and further characterization is necessary to determine if this receptor should be classified as belonging to the DAR family.

As a first step toward defining the dopaminergic transduction cascades operating in the STNS, we have cloned and characterized the two known arthropod type 1 receptors from the spiny lobster, Panulirus interruptus: D1oPan and D1jPan. In the work presented here, we define the G protein and second messenger couplings for each receptor, and examine which monoamines activate these receptors.

2. Materials and methods

2.1. Cloning and expression in a heterologous system

The three lobster DARs were cloned from nervous tissue of spiny lobster P. interruptus using a degenerate PCR strategy with conventional library screening and RACE technology as previously described (Clark et al., 2004). Full length constructs were created and inserted into a plRESneo3 vector (B.D. Biosciences Clontech, Palo Alto, CA, USA) using standard recombinant techniques. Constructs were stably expressed in HEK cells using methods previously described (Clark et al., 2004). The data have been submitted to GenBank under accession numbers DQ295790 (D1oPan) and DQ295791 (D1jPan).

2.2. Membrane preparations

Stably transfected cells were harvested with trypsin (ATCC, Manassas, VA, USA) or cell stripper (Media Tech, Herndon, VA, USA). pellets were homogenized in 20 mM HEPES (pH 7.4) containing 2 mM MgCl2, 1 mM EDTA, 2 mM 1,4-dithiothreitol (DTT), 1 μg/mL leupeptin, 1 μg/mL aprotinin, and 2 mM PMSF. The homogenate was centrifuged at 1000 ×g for 5 min. The supernatant was recovered and centrifuged at 20,000 ×g for 30 min at 4 °C. Pellets were resuspended in 20 mM HEPES (pH 7.4) containing 0.5% 3-[(3 chlamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), and 2 mM EDTA. For some experiments, samples were stored at −70 °C until assayed. Protein concentrations in each sample were determined using a BCA Protein Assay Kit (Pierce).

2.3. G protein activation assay

Agonist-induced activation of specific G proteins was determined using an assay based on a well-established, previously described protocol (Zhou and Murthy, 2004). In these experiments, individual wells of a 96-well break-apart plate (Fisher Scientific) were UV sterilized in a tissue culture hood for 15 min. At this point, wells were denoted either as blanks or coated. Coated wells received an antibody against one human Go subunit [EMD/Calbiochem catalog #371778 (Gα12), #371723 (Gα13), #371751 (Gαq), #371726 (Gα12,δ), #371732 (Gα13), or #371741 (Gα12)]. Antibodies were diluted to a concentration of 20 μg/mL in sterile phosphate-buffered saline, and 50 μL were aliquoted to separate wells. Plates were incubated on ice. After 2 h, the liquid was removed from the coated wells. Both coated and blank wells were then completely filled with blocking solution (3% BSA, 0.06% sodium azide in phosphate-buffered saline) and incubated on ice for 2 h. During this time, reactions were performed as follows. Membrane preparations from cell lines (1.5 μg/mL of protein) were incubated at 37 °C for 15 min in 10 mM HEPES (pH 7.4) containing 10 mM MgCl2, 100 μM EDTA and 10 nM GTPγS (Amersham) with or without DA. Reactions were terminated with ten volumes of termination buffer [10 mM MgCl2, 100 μM GDP, 200 mM NaCl in 100 mM Tris (pH 8.0)]. Fifty microliters of each terminated sample were then aliquoted in triplicate to both coated wells and blank wells (i.e., there are a total of six wells for each sample when measuring the activity of one G protein, nine wells for each sample when measuring the activity of two different G proteins, etc.). Plates were incubated on ice for 2 h. Wells were then rinsed three times with phosphate-buffered saline containing 0.3% Tween-20. Individual wells were placed in scintillation vials containing ScintiSafe Econo 1 (Fisher) and the radioactivity in each well was quantified with a scintillation counter. Resulting cpm from the blank wells were averaged and used as a measure of non-specific binding. The nonspecific binding was subtracted from the average cpm obtained from the coated wells. Data are expressed as cpm/μg of protein. 2.4. cAMP assays

cAMP levels were measured as previously described (Clark et al., 2004). Briefly, 1 × 10⁵ cells were plated in 35 mm dishes and grown to confluence. Cells were washed with 1 mL of medium and preincubated at 37 °C for 10 min in the presence of the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthline (2.5 mM) (Sigma). In some cases, cells were incubated an additional 30 min at 37 °C with or without forskolin (2.5 μM) and varying concentrations of DA. In some experiments, cells were pretreated for 24 h with pertussis toxin (PTX, Calbiochem) or 15 min with 1-O-Octadecyl-2-O-methyl-rac-glycerol-3-phosphorylcholine (Et-18-OCH3, Calbiochem). The medium was removed and 0.5 μL of 0.1 M HCl (Sigma) with 0.8% Triton X-100 (Sigma) was added to the plates. After a 30 min incubation at room temperature, the lysate was removed from the plates and centrifuged for 2 min. The supernatant was collected and assayed for cAMP levels using a direct cAMP enzyme immunoassay kit (Assay Designs, Inc.) according to the manufacturer’s instructions. Protein concentrations in each sample were determined using a BCA Protein Assay Kit (Pierce). Data are expressed as picomoles of cAMP/milligram of protein.
2.5. Statistical analyses and curve fitting

Student t-tests were performed with Excel software. Curve fitting and Kruskal–Wallis (ANOVA on ranks) tests were performed with Prism (GraphPad Software, San Diego, CA, USA, www.graphpad.com). In all cases, statistical significance was determined as $p<0.05$.

3. Results

3.1. The family of dopamine receptors is conserved across different classes of arthropods

To begin to elucidate the dopaminergic systems in the STNS, we cloned the two known arthropod type-one DARs from *P. interruptus*: $D_{1\alpha\text{Pan}}$ and $D_{1\beta\text{Pan}}$. The $D_{1\alpha\text{Pan}}$ receptor is orthologous to the *Drosophila* receptor DAMB/DopR99B (Feng et al., 1996; Han et al., 1996) and the $D_{1\beta\text{Pan}}$ receptor is orthologous to the *Drosophila* receptor Dmdop1/Dda1 (Blenau et al., 1998; Gotzes et al., 1994; Sugamori et al., 1995). Fig. 1 illustrates that orthologs show high homology. Paired alignments of spiny lobster and *Drosophila* $D_{1\alpha}$ and $D_{1\beta}$ orthologs revealed 44% and 37% amino acid identity over the entire protein, respectively. Most differences across species occur in the amino and carboxy termini and intracellular loop 3, which is typical for GPCRs (Clark et al., 2004; Sosa et al., 2004). Indeed, the idea of divergent termini is emphasized by the fact that the gene for the $D_{1\beta}$ receptor is alternately spliced to produce two proteins with different amino termini, $D_{1\beta1.1\text{Pan}}$ and $D_{1\beta2.2\text{Pan}}$ (Table 1; Fig. 1). Interestingly, and contrary to the idea that the carboxy termini often diverge, both $D_{1\beta\text{Pan}}$ orthologs end in a conserved PDZ domain. However, we have not performed an exhaustive search for alternate splice forms, and it is possible that there are additional alternately spliced exons for both $D_{1\alpha\text{Pan}}$ and $D_{1\beta\text{Pan}}$ receptors.

3.2. $D_{1\alpha\text{Pan}}$ couples with Gs in HEK cells to produce an increase in $[cAMP]$.

We next characterized receptor couplings in a heterologous expression system. When bound by ligand, activated DARs function as guanine nucleotide exchange factors (GEFs), causing inactive heterotrimeric G proteins to exchange GDP for GTP. The trimeric G protein then dissociates into $G_{\alpha}$ and $G_{\beta\gamma}$ subunits, each of which interacts with downstream effectors (Cabrera-Vera et al., 2003). Since vertebrate and insect $D_{1}$ receptors preferentially couple with $G_{s}$ to stimulate adenylyl cyclase and increase cAMP levels (Feng et al., 1996; Fig. 1. The DAR family is conserved across arthropods. The *Panulirus* (L) and *Drosophila* (F) DAR orthologs are aligned. Amino acids that are identical in fly and lobster orthologs are highlighted for each pair of DARs. Black bars approximate the seven transmembrane regions. The point of alternate splicing on lobster $D_{1\beta1\text{Pan}}$ is indicated by a black arrowhead. The accession numbers are as follows: LD$D_{1\alpha\text{Pan}}$, DQ295790; FD$D_{1\alpha\text{Pan}}$, U34383; LD$D_{1\beta1.1\text{Pan}}$, DQ295791; FD$D_{1\beta2.2\text{Pan}}$, X77234.1.)
Han et al., 1996; Neve et al., 2004), we predicted that the D1αPan receptor should do likewise. To test this prediction, full-length D1αPan constructs were assembled using standard recombinant techniques. The constructs were then stably expressed in HEK cells, and the resulting cell line, HEK D1αPan, was assayed for changes in G protein activity and cAMP levels.

We first developed and performed a “G protein activation assay” based on minor modifications to a previously described protocol (Zhou and Murthy, 2004). In this assay, the wells of a break-apart 96-well plate are pre-coated with commercially available antibodies against the various human G proteins. Membrane fractions of a human cell line stably expressing a membrane preparations were measured in the absence (open bar) vs. the presence (filled bar) of 10^−8 M DA for eight G proteins: Gs, Gq, Gz, Giα, Giβ, Giγ, Go, G12. Data represent the mean±S.E.M., n=3.

Fig. 4. The D1αPan receptor couples with Gs and Gz. G protein activities in HEK D1αPan membrane preparations were measured in the absence (open bar) vs. the presence (filled bar) of 10^−5 M DA for eight G proteins: Gs, Gq, Gz, Giα, Giβ, Giγ, Go, G12. Data represent the mean±S.E.M., n≥3. Statistically significant differences in the activity of a given G protein are indicated with an asterisk (p<0.05).
3.3. \(D_{1\beta}\text{Pan}\) couples with \(G_s\) and \(G_z\) in HEK cells, resulting in increased \([cAMP]\)

Insect orthologs of the arthropod \(D_{1\beta}\) receptor have previously been shown to positively couple with adenyl cyclase, suggesting that this receptor couples to \(G_s\) (Blenau et al., 1998; Gotzes et al., 1994; Sugamori et al., 1995). We performed the G protein activation assay on the HEKD\(1\beta\text{Pan}\) cell lines to determine \(D_{1\beta}\text{Pan}\) receptor-G protein coupling. Fig. 4 indicates that the \(D_{1\beta}\text{Pan}\) receptor couples with multiple G proteins. A 15 min exposure to 10 \(\mu\text{M}\) DA produced a ~5-fold increase in \(G_{\alpha}\) activity \((p<0.002)\). DA also produced a significant 1.6-fold increase in the activity of \(G_{\alpha}\) \((p<0.004)\), a PTX insensitive member of the \(G_{\alpha}\) family that negatively couples with adenyl cyclase to reduce cAMP levels (Ho and Wong, 2001). The stimulation of \(G_{\alpha}\) was roughly 3 times larger than that of \(G_z\). The human \(D_{1\beta}/D_3\) receptor has also been shown to couple with \(G_s\) and \(G_z\) in \(G_{12/13}\) cells (Sidhu et al., 1998).

Fig. 3 indicates that the \(D_{1\beta}\text{Pan}\) receptor couples with G proteins that regulate adenyl cyclase in opposing directions (i.e., \(G_s\) increases adenyl cyclase activity while \(G_z\) decreases adenyl cyclase activity). Since DA induced \(G_s\) activity was three times larger than DA induced \(G_z\) activity, we predicted that DA should elicit a net increase in cAMP in HEKD\(1\beta\text{Pan}\) cell lines. Fig. 5 illustrates that stable cell lines expressing different isoforms of the full-length lobster \(D_{1\beta}\) receptor (HEK \(D_{1\beta,1\text{Pan}}\) and HEK \(D_{1\beta,2\text{Pan}}\)) show a dose dependent increase in cAMP in response to increasing concentrations of DA, with EC\(_{50}\) values between 1 and \(1.4 \times 10^{-6}\). In addition, the \(D_{1\beta}\text{Pan}\) isoforms appear to be constitutively active. As shown in Fig. 5, in the absence of DA cAMP levels were significantly higher in HEK \(D_{1\beta}\text{Pan}\) cells relative to parental cells \((p<10^{-5}\) for both isoforms). Thus, both isoforms of the \(D_{1\beta}\text{Pan}\) receptor display agonist independent activity like the mammalian \(D_{1\beta}/D_3\) receptor (Demchyshyn et al., 2000). The data do not indicate whether coupling with \(G_z\) was also constitutive.

3.4. Dopamine activates lobster type 1 DA-Rs

Monoamines act as circulating neurohormones and neurotransmitters in the STNS. Five endogenous biogenic amines can modulate STNS neurons: dopamine, serotonin (5-HT), tyramine, octopamine and histamine. In some cases, it has been reported that arthropod DARs can respond to multiple monoamines in heterologous expression systems (Hearn et al., 2002). Activation of lobster DARs by multiple monoamines could have important implications for monoaminergic signal transduction in the STNS. We therefore asked which of the endogenous monoamines could activate \(D_{1\alpha}\text{Pan}\) and \(D_{1\beta}\text{Pan}\) receptors.

Levels of cAMP were measured in three cell lines (HEK, HEKD\(1\alpha\text{Pan}\) and HEKD\(1\beta,2\text{Pan}\)) before and after exposure to one of the five monoamines. Fig. 6 illustrates that DA activation of \(D_{1\alpha}\text{Pan}\) and \(D_{1\beta}\text{Pan}\) produced significant, approximately 5.3- and 3.6-fold increases in cAMP levels in the HEK \(D_{1\alpha}\text{Pan}\) and HEKD\(1\beta\text{Pan}\) cell lines, respectively, but had no significant effect on the parental HEK cell line. Thus, the heterologously expressed receptors are responsible for the DA-induced increase in cAMP in HEKD\(1\alpha\text{Pan}\) and HEKD\(1\beta\text{Pan}\) cell lines.

At a concentration of 1 mM, octopamine and tyramine had no significant effect on any of the three cell lines examined. On the other hand, Fig. 6 demonstrates that 1 mM 5-HT produced a significant, roughly 3-fold increase in cAMP in HEK cells, suggesting that the parental cell line contains endogenous 5-HT receptors that are positively coupled to adenyl cyclase, as has been previously reported (Johnson et al., 2003b). The same increase was observed in the HEKD\(1\alpha\text{Pan}\) and HEKD\(1\beta\text{Pan}\) cell lines. The 5-HT induced cAMP increases in all three cell lines were not significantly different from one another, suggesting that the responses are due to the endogenous 5-HT receptors and not the heterologously expressed DARs.

Similarly, the parental HEK cell line appears to express endogenous histamine receptors, as 1 mM histamine produced a
significant, approximately 3-fold increase in cAMP in HEK cells. This increase was also observed in the HEKD_{1αPan} and HEKD_{1βPan} cell lines. The responses in the three cell lines were not significantly different from one another, suggesting that they were due to the endogenous histamine receptors and not the heterologously expressed DARs. In summary, DA activates D_{1αPan} and D_{1βPan} receptors, but serotonin, histamine, octopamine, and tyramine do not.

4. Discussion

The work presented here represents the first step toward defining the molecular underpinnings of dopaminergic neuro-modulation in the STNS. We have shown that the structure and function of the spiny lobster DARs, D_{1αPan} and D_{1βPan} are conserved across class and phyla. D_{1αPan} couples with Gs to increase cAMP while D_{1βPan} couples with Gs and Gz to produce a net increase in cAMP. Moreover, of the 5 biogenic amines tested, only DA activated these receptors.

In all systems, dopaminergic effects are mediated through GPCRs that interact with G proteins. Both G proteins and GPCRs are well conserved across vertebrate/invertebrate lines, especially with regard to interaction domains. Indeed, Table 2 shows that the C-terminal domain of the G protein, which physically interacts with the GPCR, is identical for homologous G proteins in lobsters and humans! There are 6 G\_\alpha proteins in arthropods: G\_\alpha_s, G\_\alpha_f (Gs-like at the DNA level), G\_\alpha_q, G\_\alpha_i, G\_\alpha_o, G\_\alpha_{12} (http://flybase.net/). Three of the G proteins have been cloned from lobster (McClintock et al., 1992, 1997; Xu et al., 1997). As shown in Table 2, the C-termini of lobster G\_\alpha_s, G\_\alpha_q and G\_\alpha_i are completely conserved with their human homologs. Thus, it is reasonable to probe the coupling specificity of spiny lobster GPCRs in human cell lines.

Traditionally, DARs were thought to couple only with Gs to increase [cAMP]_{i} and Gi/o to decrease [cAMP]_{i}; however, recent studies in heterologous expression systems, including the one presented here, suggest that DAR-G protein coupling can be much more diverse (Sidhu and Niznik, 2000). For example, D_{1}/D_{1A} can couple with Gs and Go in reconstitution experiments or in overexpression experiments with rat pituitary GH2C1 cells (Kimura et al., 1995a,b). D_{1B}/D_{2} can couple with Gs and Gz in GH2C1 cells (Sidhu et al., 1998), or with Gs and G12 in immortalized rat renal proximal tubule cells (Zheng et al., 2003). D_{2} receptors couple with multiple members of the Gi/o family (Banihashemi and Albert, 2002; Ghahremani et al., 1999; Obadiah et al., 1999). When expressed in CHO cells, human D_{3} receptors may couple with the Gi/o and Gq families (Newman-Tancredi et al., 1999). Studies in native systems also suggest non-classical coupling such that D_{1} receptors may couple with Gs and Gq in the mammalian and C. elegans CNS (Chase et al., 2004; O’Sullivan et al., 2004; Undie et al., 2000; Wersinger et al., 2003; Zhen et al., 2004).

It is interesting that the lobster D_{1αPan} Receptor, like the human D_{1B}/D_{2} receptor, can couple with both Gs and Gz. We do not know the proteins that interact with the lobster DARs to facilitate multiple couplings. Both post-translational modifications and receptor-interacting proteins can cause a receptor to switch G protein coupling. When phosphorylated by protein kinase A, the β_{3}-adrenergic receptor switches its coupling from Gs to Gi (Baillie et al., 2003; Daaka et al., 1997). Receptor coupling can also be extended by receptor-interacting proteins, like calceyon, which regulates receptor cross-talk and allows D_{1} receptors to switch coupling between Gs and Gq (Lezcano et al., 2000). It is not obvious why this receptor would couple to multiple cascades that have opposing effects on cAMP. It is possible that the population of cells is heterogeneous so that there is only one type of coupling per cell. On the other hand, when simultaneously activated, opposing cascades in a single cell may be highly localized so that microdomains of cAMP gradients are created (Zaccolo and Pozzan, 2002). Alternatively the cascades may function with different kinetics and interact to generate feedback loops and/or multiphasic responses.

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References


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<td>Comparison of G protein C-termini across species</td>
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<td>G_\alpha_s</td>
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H = human, L = lobster, F = fly.


