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Comparative pharmacology of two D1-like dopamine receptors cloned from the silkworm *Bombyx mori*

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ABSTRACT

Dopamine (DA) is a physiologically important biogenic amine in insect peripheral and nervous tissues. We recently cloned two DA receptors (BmDopR1 and BmDopR2) from the silkworm *Bombyx mori* and identified them as D1-like receptors, which activate adenylate cyclase to increase intracellular cAMP levels. In this study, these two receptors were stably expressed in HEK-293 cells, and the dose-responsiveness to DA and their pharmacological properties were examined using cAMP assays. BmDopR1 showed a dose-dependent increase in cAMP levels at DA concentrations up to 10^{-7} M with EC_{50} of 3.30 nM, while BmDopR2 required 10^{-6} M DA for activation. In BmDopR1-expressing cells, DA at 10^{-6}–10^{-4} M induced 30–50% lower cAMP production than 10^{-7} M DA. BmDopR2-expressing cells showed a standard sigmoidal dose–response, with maximum cAMP levels attained with 10^{-5}–10^{-4} M DA and EC_{50} of 1.30 μM. Both receptors had similar agonist profiles, and the typical vertebrate D1-like receptor agonist SKF-38393 was ineffective. Experiments with antagonists revealed that BmDopR1 exhibits D1-like features. However, the pharmacology of BmDopR2 was distinct from D1-like receptors; the typical vertebrate D1-like receptor antagonist SCH-23390 was less potent than the nonselective antagonist flupenthixol and the D2-like receptor antagonist chlorpromazine. The rank order of activities of several antagonists for BmDopR1 and BmDopR2 was more similar to that of *Drosophila melanogaster* DA receptors than *Apis mellifera* DA receptors. These data suggest that DA receptors could be potential targets for specific insecticides or insectistatics.

1. Introduction

Biogenic amines found in the peripheral and nervous tissues of insects function as neurotransmitters, neuromodulators and neurohormones, and mediate diverse physiological events (Evans, 1980; Bicker and Menzel, 1989; Scheiner et al., 2006). Catecholamines such as adrenaline, noradrenaline, and dopamine (DA) are typical biogenic amines in vertebrates; however, in invertebrates such as insects, DA is thought to be the only catecholamine with physiological significance (Osborne, 1996; Roeder, 2002). DA exerts its effects by interacting with specific membrane receptors. In vertebrates, five distinct seven-transmembrane G-protein-coupled DA receptors (D1–D5) have been cloned, and classified into D1-like (D1 and D5) and D2-like (D2–D4) receptors on the basis of their biochemical, pharmacological, and structural properties (Missale et al., 1998). These two classes of DA receptors are linked to distinct signal transduction cascades and have opposing effects on adenylate cyclase; D1-like receptors activate adenylate cyclase, whereas D2-like receptors inhibit it (Missale et al., 1998; Vallone et al., 2000).

Two D1-like receptors and one D2-like receptor, which are classified in a similar fashion to vertebrate DA receptors, have been cloned and characterized from *Drosophila melanogaster* (Gotzes et al., 1994; Sugamori et al., 1995; Feng et al., 1996; Han et al., 1996; Hearn et al., 2002) and *Apis mellifera* (Blenau et al., 1998; Humphries et al., 2003; Beggs et al., 2005), and one of the D1-like receptor cDNAs has been cloned from the swallowtail butterfly *Papilio xuthus* (Ono and Yoshikawa, 2004). Information on the physiological roles of these DA receptors has been gradually accumulating.

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The *Drosophila* D1-like receptor, dDA1, is required in mushroom body neurons for aversive and appetitive learning (Kim et al., 2007). The *Drosophila* D2-like receptor, DD2R, regulates locomotor activity (Draper et al., 2007). In young worker bees, changes in DA receptor expression in the brain by queen mandibular pheromone are involved in behavioral modulation and motor control (Beggs et al., 2007). In connection with DA receptors, a G-protein-coupled receptor that responds to DA and the insect hormones, ecdysone and 20-hydroxyecdysone, has been cloned from *Drosophila* and characterized (Srivastava et al., 2005).

We recently cloned orthologs of the two D1-like receptors from the silkworm *Bombyx mori* and named them BmDopR1 and BmDopR2 (Mitsumasu et al., 2008). Although the amino acid homology score between them was low (30%), both receptors showed an identical function, i.e., remarkable cAMP production by DA application in a heterologous system. The production occurred DA-selectively; other biogenic amines (octopamine, tyramine, and serotonin) did not activate both receptors in the same batch. BmDopR1 was activated at a lower concentration of DA than BmDopR2. In addition, DA increased levels of cAMP to a greater extent by interaction with BmDopR1 compared to BmDopR2, implying that DA has higher potency and efficacy when interacting with BmDopR1. However, the dose-responsiveness of BmDopR1 and BmDopR2 to DA and their pharmacology remain unexamined. Comparison of the biochemical and pharmacological properties of *Bombyx* DA receptors would enable identification of subtype-specific agonists or antagonists, which would be useful tools to examine the involvement of DA receptor subtypes in the regulation of various physiological and behavioral events in insects. In addition, if the pharmacology of *Bombyx* DA receptors is distinct from that of other insects and vertebrates, agonists or antagonists that act at *Bombyx* DA receptors may prove useful in developing insecticides or insectistatics that specifically act at lepidopteran DA receptors.

Herein, we report the biochemical and pharmacological differences between the *Bombyx* DA receptors estimated from the results of cAMP assays. Furthermore, we pharmacologically compared each *Bombyx* DA receptor with vertebrate D1-like receptors or their *Drosophila* and *Apis* orthologs. These comparative pharmacological data might aid in the development of species-specific insecticides or insectistatics that target DA receptors.

2. Materials and methods

2.1. Pharmacological ligands and other chemicals

DA·HCl was purchased from Nacalai Tesque, Inc. (Kyoto, Japan). (±)-2-Amino-6,7-dihydroxy-1,2,3,4-tetrahydronaphthalene (6,7-ADTN)·HBr, (*R*)-(−)-apomorphine·HCl·1/2H_2O, 2-bromo-α-ergocryptine (bromocriptine)·CH_4SO_3, (*R*)-(+)-SKF-38393·HCl, *cis*-(*Z*)-flupenthixol·2HCl, (*R*)-(+)-SCH-23390·HCl, spiperone, and (+)-butaclamol·HCl were from Sigma–Aldrich (St. Louis, MO, USA). Chlorpromazine·HCl was obtained from RBI Research Biochemicals, Inc. (Natick, MA, USA), and [^{3}H]cAMP (17 Ci/mmol) was purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO, USA). Theophylline and other general reagents were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

2.2. Construction of expression vectors

cDNAs for BmDopR1 and BmDopR2 (accession nos. AB362162 and AB162716) were cloned in our previous study (Mitsumasu et al., 2008). The open reading frames (ORFs) were amplified by PCR using DNA polymerase Ex Taq (Takara Shuzo Co., Ltd., Shiga, Japan), with the plasmid clones as templates. The amplified products for the ORFs of BmDopR1 and BmDopR2 were ligated into the BamHI and KpnI/XbaI sites of the expression vector pcDNA3 (Invitrogen, Carlsbad, CA, USA), respectively, to produce the recombinant pcDNA3-BmDopR1 and -BmDopR2. Each insertion was confirmed by DNA sequencing.

2.3. Cell culture and transfection

HEK-293 cells were grown in Dulbecco's modified Eagle's medium (D-MEM; Invitrogen) supplemented with 10% fetal bovine serum (FBS; Invitrogen) at 37 °C and 5% CO_2. Cells (2×10^5) suspended in D-MEM containing 10% FBS were plated on 35-mm dishes 1 day before transfection. The attached cells were transfected with pcDNA3-BmDopR1 and -BmDopR2 using 4 μl of 2 mg/ml Lipofectamine (Invitrogen) in 1 ml of Opti-MEM I reduced serum medium (Invitrogen). After incubation for 5 h at 37 °C and 5% CO_2, D-MEM containing 20% FBS (1 ml) was added without removing the transfection mixture, and the cells were allowed to incubate for 1 day. The cells were then washed two times with 1 ml of Dulbecco's phosphate-buffered saline (D-PBS; Invitrogen) and reseeded at a low density in fresh medium. The following day, the antibiotic Geneticin (G418; Sigma–Aldrich) was added at 1 mg/ml to the culture medium to select cells stably expressing BmDopR1 or BmDopR2. The cell selection was performed for approximately 3 weeks, and the medium containing G418 was replenished every 4–5 days. The selected stable HEK-BmDopR1 and HEK-BmDopR2 cells were collected as polyclonal populations and stored frozen in medium-DMSO (Invitrogen) at −80 °C. HEK-mock cells were constructed as G418-resistant polyclonal cells by stable transfection with empty pcDNA3.

2.4. cAMP assays

HEK-mock, -BmDopR1, and -BmDopR2 cells were thawed rapidly at 37 °C and grown in the presence of G418 at 1 mg/ml. Cells (5×10^5) suspended in D-MEM containing 10% FBS were plated on 35-mm dishes and allowed to adhere for at least 16 h. The attached cells were washed once with 1 ml of D-PBS and preincubated in 1.8 ml of D-PBS containing 5 mM theophylline for 10 min at 37 °C. After preincubation, a 200-μl aliquot of D-PBS containing each ligand was added to the cells. Antagonists were added along with DA. After gently stirring the dishes, the culture was incubated for 10 min at 37 °C. The reaction was stopped by aspiration of the medium and addition of 300 μl of ice-cold acidic ethanol (1 M HCl/ethanol, 1/100). The cells in the ethanol solution were collected and homogenized in 1.5-ml microtubes. The sample was incubated for 5 min at room temperature, and the debris was removed by centrifugation at 14,000 × *g* for 5 min. The supernatant was evaporated to dryness by vacuum centrifugation for 1 h at 45 °C. The residue was suspended in 120 μl of 50 mM Tris–HCl buffer (pH 7.4) with 4 mM EDTA and centrifuged at 14,000 × *g* for 5 min. The supernatant was used to determine intracellular cAMP levels. The cAMP levels were determined with a [^{3}H]cAMP assay system using cAMP-binding protein (Munirathinam and Yoburn, 1994). The radioactivity was measured using a liquid scintillation counter. EC_{50} values were estimated by the Probit method.

3. Results

3.1. Effects of DA on intracellular cAMP levels in HEK-BmDopR1 and -BmDopR2 cells

The dose–response of DA on intracellular cAMP levels was examined in the heterologous DA receptor expressing systems. In HEK-BmDopR1 cells (Fig. 1A), treatment with 10^{-8} M DA elevated