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Two amino acids in each of D_1 and D_2 dopamine receptor cytoplasmic regions are involved in D_1–D_2 heteromer formation

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ABSTRACT

D_1 and D_2 dopamine receptors exist as heteromers in cells and brain tissue and are dynamically regulated and separated by agonist concentrations at the cell surface. We determined that these receptor pairs interact primarily through discrete amino acids in the cytoplasmic regions of each receptor, with no evidence of any D_1–D_2 receptor transmembrane interaction found. Specifically involved in heteromer formation we identified, in intracellular loop 3 of the D_2 receptor, two adjacent arginine residues. Substitution of one of the arginine pair prevented heteromer formation. Also involved in heteromer formation we identified, in the carboxyl tail of the D_1 receptor, two adjacent glutamic acid residues. Substitution of one of the glutamic acid pair prevented heteromer formation. These amino acid pairs in D_1 and D_2 receptors are oppositely charged, and presumably interact directly by electrostatic interactions.

1. Introduction

Family A G protein coupled receptors (GPCRs) form heteromers [1–3]. We reported that D_1–D_2 receptor heteromers exist in brain and cultured neurons [4,5]. We showed receptor activation within D_1–D_2 heteromers generated a Gq-mediated calcium signal [4,6,7]. We have determined that D_1–D_2 heteromers were subject to conformational changes and separation by dopamine or receptor-selective agonists [8]. We also reported that the D_1 and D_2 receptor heteromers reform at the cell surface when the agonist was removed [8]. These data provided evidence of the fate of a heteromer following agonist activation and demonstrated a unique regulation of GPCRs at the cell surface. However, many fine structural details of how D_1–D_2 heteromers dynamically interact remain unknown. In this report we have determined the precise amino acid interactions maintaining D_1 and D_2 receptors in a D_1–D_2 receptor complex. Our ultimate goal is the understanding of the physiological relevance of GPCR:GPCR heteromers, one of the leading questions in the GPCR field.

Progress in the fundamental area of GPCR oligomer structural investigation has been hampered by the lack of decisive methods for determining the interacting heteromer interface. We overcame technical challenges by the following process: a nuclear localization sequence (NLS) was inserted into the D_2 receptor. Strategic placement of the NLS rendered this D_2-NLS receptor conformationally sensitive, so that interacting ligands retained the receptor at the cell surface [9]. D_2-NLS and the D_1 receptors were coexpressed and following ligand removal, the D_2-NLS receptor translocated with the D_1 receptor from the cell surface. We demonstrated that as the D_2-NLS receptor translocated with the D_1 receptor this provided a tool to study receptor:receptor dynamic interactions in a cell [9]. By this strategy we sought to reveal the structural basis for the D_1–D_2 receptor interaction. By co-expressing D_2-NLS and D_1 receptors the contributions of various cytoplasmic regions of these receptors to heteromer formation was investigated.

In this report, we have determined the precise amino acids in the cytoplasmic regions of both the D_1 and D_2 receptors involved in their heteromeric interactions. Activation of the heteromer contributes to conformational changes in the receptors within the oligomer. We have now identified these residues affected by agonist induced conformational changes. Also we identified that changing a single amino acid in the intracellular loop 3 of the D_2 receptor or in the carboxyl tail of the D_1 receptor prevented D_1–D_2 heteromer formation.

2. Materials and methods

2.1. Fluorescent proteins

cDNA sequences encoding GFP, RFP were obtained from Clontech (Palo Alto, CA), and the receptor constructs generated as described [9]. The YFP vector was obtained from BD Biosciences.

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2.2. Cell culture

HEK cells grown to confluence on 60 mm plates in minimum essential medium (MEM), and were transfected with 0.5–2 μg cDNA using Lipofectamine (Life Technologies, Rockville MD).

2.3. Microscopy

Live cells expressing GFP, RFP and YFP fusion proteins were visualized with a LSM510 Zeiss confocal laser microscope. In each experiment 5–8 fields, containing 50–80 cells per field were evaluated and the entire experiment was repeated several times (n = 3–5).

2.4. DNA constructs

All the DNA encoding the GPCRs were human origin. Sequences encoding GPCRs were cloned into plasmids pEGFP, as described previously [9]. The D_1 carboxyl tail DNA PCR product, containing no stop codon was subcloned into vector pYFP-N1 (BD Biosciences) at EcoR1 and Kpn1 and inframe with the start codon of YFP.

2.5. Receptor constructs

The D_1 and D_2 receptors were prepared using the Quickchange mutagenesis kit (Stratagene) according to the manufacturer's instructions, and as described [9]. Receptor DNA was subjected to PCR as previously reported [9]. The reaction mixture consisted of: H_2O (32 μl), 10× Pfu buffer (Stratagene) (5 μl), dNTP (10 mM, 5 μl), DMSO (5 μl), oligonucleotide primers (100 ng, 1 μl each), DNA template (100 ng), Pfu enzyme (5U). Total volume 50 μl. PCR conditions, one cycle at 94 °C for 2 min, 30–35 cycles at 94 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min, per cycle, and then one cycle at 72 °C for 5 min. The NLS sequence was inserted into DNA encoding the D_1 and D_2 dopamine receptors by PCR [8].

2.6. Membrane preparation

Cells expressing D_2-NLS or D_1-NLS were washed with phosphate-buffered saline, resuspended in hypotonic lysis buffer (5 mM Tris–HCl, 2 mM EDTA, 5 μg/ml leupeptin, 10 μg/ml benzamide, 5 μg/ml soybean trypsin inhibitor, pH 7.4), and homogenized by Polytron (Brinkmann Instruments). The homogenate was centrifuged to pellet unbroken cells and nuclei. The supernatant centrifuged at 40,000g to obtain a membrane pellet.

2.7. Radioligand binding assays

Competition binding assays were performed as described previously [1,3]. Briefly, for competition experiments, 20–25 μg of membrane was incubated with 1nM [^{3}H]-raclopride (for D_2) or [^{3}H]-SCH23390 (for D_1) (NEN Life Science Products) and increasing concentrations of competing drug. The reaction volume was 0.5 ml, and the binding buffer consisted of 50 mM Tris–HCl, 5 mM EDTA, 1.5 mM $CaCl_2$, 5 mM $MgCl_2$, 5 mM KCl, and 120 mM NaCl, pH 7.4. Nonspecific binding was defined using 1 μM (+)-butaclamol (Research Biochemicals International, Hercules, CA). Binding reactions were incubated at room temperature for 2 h to reach equilibrium. Bound radioligand was then isolated from free by rapid filtration through a Brandel 48-well harvester using Whatman GF/C filters. Data were analyzed using nonlinear least squares regression equations on the curve-fitting computer program Prism (Graphpad).

3. Results

3.1. Binding and expression properties of the D_2-NLS receptor and D_1-NLS receptors

The incorporation of NLS into the D_2 receptor did not alter the binding properties, with preserved agonist-detected high affinity and low affinity states, indicative of intact receptor-G protein coupling. The D_2 receptor had a K_{High} value of 1.51×10^{-9} M and K_{Low} of 6.67×10^{-6} M for quinpirole. Similarly the D_2-NLS receptor had a K_{High} value of 3.22×10^{-9} M and K_{Low} of 4.16×10^{-6} M for quinpirole [9].

The incorporation of the NLS into the D_1 receptor did not alter the binding pocket of the receptor, with preserved agonist- detected high affinity and low affinity states, indicative of intact receptor-G protein coupling and ligand affinities. The D_1-NLS receptor had a K_{high} value of 4.17×10^{-9} M and K_{Low} of 1.19×10^{-7} M detected by agonist SKF 81297 not different from unmodified D_1 receptor [9].

3.2. Identification of the D_2 dopamine receptor amino acids involved in D_1–D_2 heteromer formation

We wished to determine if amino acids located in the cytoplasmic loops of the D_2 receptor were involved in forming heteromeric complexes with the D_1 receptor. The D_2 receptor has an unusual GPCR structure in having no significant carboxyl tail, as the carboxyl tail terminates with the palmitoylated cysteine [10]. There are two forms of the D_2 dopamine receptor, namely D_2 long and D_2 short, differing by a 29 amino acid insert in ic3, located thirty amino acids from transmembrane 5 (TM5; Fig. 1) [11]. The very large intracellular D_2 receptor third loop (intracellular loop 3, ic3) contains ~160 amino acids, this region comprises 40% of the total receptor structure, Fig 1. The D_2-NLS long receptor with a fully intact ic3 and the D_1 receptor are shown co-expressed in Fig. 2A, with significant co-translocation indicating robust heteromer formation.

In our strategy, initially working with the D_2 long receptor, we prepared a series of D_2 receptor constructs with deletions contained in this third loop (outlined in Table 1 and Fig. 1). Each of these ic3 receptor constructs of the D_2-NLS receptor were co-expressed with the D_1 receptor. In each case D_1–D_2 heteromerization was monitored by the ability of these D_2-NLS receptors to enable transportation of the D_1 receptor from the cell surface to the cytoplasm and nucleus. We first determined that a large deletion, L1, of 72 amino acids (Table 1 and Fig 1), from the carboxyl terminal half of D_2 receptor ic3 had no effect on D_1–D_2 heteromer formation, these receptors translocated together (Fig. 2B). However, another D_2 receptor construct, L2, with 72 amino acids deleted from the amino terminal half of ic3 (Table 1 and Fig. 1) failed to show D_1–D_2 receptor co-translocation, and hence failed to form D_1–D_2 receptor heteromers, Fig. 2C. Thus data from the L2 construct indicated that amino acids maintaining heteromer formation were likely contained in this region. To locate the critical amino acids, portions of this ic3 L2 region were serially deleted to identify regions involved in the interaction with the D_1 receptor.

We divided the L2 region into two parts, L3 (24 amino acids) and L4 (19 amino acids, Fig. 1), not including the 29 amino acid insert of the D_2 long receptor. The construct L4 formed D_1–D_2 receptor heteromers while construct L3 did not (L3 shown in Fig 2D), thus the region involved in heteromer formation was contained in the 24 amino acids of construct L3. The L3 region was divided in two equal parts, with constructs L5 and L6. Only construct L6 failed to form heteromers with the D_1 receptor and this region of 12 amino acids was further divided in two equal parts, in