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D_5 dopamine receptor carboxyl tail involved in D_5–D_2 heteromer formation

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ABSTRACT

We have demonstrated that D_5 and D_2 dopamine receptors exist as heteromers in cells, and determined these receptor interact through amino acids in the cytoplasmic regions of each receptor. Specifically involved in heteromer formation we identified in the carboxyl tail of the D_5 receptor three adjacent glutamic acid residues, and in intracellular loop 3 of the D_2 receptor two adjacent arginine residues. Any pairing of these three D_5 receptor glutamic acids were sufficient for heteromer formation. These identified residues in D_5 and D_2 receptors are oppositely charged and likely interact by electrostatic interactions.

1. Introduction

Family A G protein coupled receptors (GPCRs) form heteromers [1–3]. We have reported that dopamine D_1–D_2 receptor heteromers exist in brain and cultured neurons [4,5]. These heteromers were subject to conformational changes and separation by agonists [6], the heteromers reformed at the cell surface when the agonist was removed [6]. Identifying specific amino acids involved in GPCR heteromer formation has been hampered by the lack of decisive methodologies. Using our process of inserting a nuclear signal (nls) into a GPCR [7] we have identified residues involved in forming heteromers. We reported that the D_1 and D_2 heteromers interact by specific residues in the cytoplasmic regions. In intracellular loop 3 (ic3) of the D_2 receptor, two arginine residues (274-RR) form an electrostatic interaction with vicinal glutamic residues (404-EE) in the carboxyl tail (c-tail) of the D_1 receptor [8]. We also recently identified cytoplasmic residues involved in mu-delta opioid heteromers [9].

Previously we demonstrated heteromerization between the D_5 and D_2 receptors, our FRET analysis showed D_5 and D_2 receptors formed a heteromeric complex [10]. The D_1 and D_5 dopamine receptors share extensive overall homology (80%), however these receptors have negligible homology in their long c-tails. We questioned if D_5 and D_2 heteromers also form by electrostatic interactions between the D_2 ic3 and D_5 c-tail. In this report we have determined the specific amino acids in the cytoplasmic regions of D_5 and D_2 receptors involved in heteromer interactions. We demonstrated that changing the identified cytoplasmic amino acids prevented D_5–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 [7].

2.2. Cell culture

HEK cells grown on 60 mm plates in minimum essential medium (MEM), were transfected with 0.5–2 μg cDNA using Lipofectamine (Life technologies, Rockville MD). Dopamine antagonist (+)butaclamol when used, was added to cells and cells visualized by confocal microscopy.

2.3. Microscopy

Live cells expressing GFP, and RFP 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).

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2.4. DNA constructs

All the DNA encoding the GPCRs were human origin. Sequences encoding GPCRs were cloned into plasmids pEGFP, as described previously [7,11] . The D_5 carboxyl tail DNA PCR product, containing no stop codon was subcloned into vector RFP (BD Biosciences) at EcoR1 and Kpn1 and inframe with the start codon of RFP.

2.5. Receptor constructs

The D_5 receptor constructs were prepared using the Quickchange mutagenesis kit (Stratagene) according to the manufacturer's instructions, and as described [7,11] . Receptor DNA was subjected to PCR as previously reported [7]. 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 (5 U). 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 D_2-nls and D_1-nls receptor construct was prepared as previously described [7].

3. Results

3.1. Identification of the D_5 dopamine receptor amino acids involved in D_5–D_2 heteromer formation

The D_5 receptor has an extensive c-tail, extending ~93 amino acids from the palmitoylated cysteine, Fig. 1, (consists of 26% of the total D_5 receptor, the D_1 receptor c-tail is 95 amino acids in length). There is negligible homology shared between the D_1 and D_5 receptors throughout their c-tail regions.

We incorporated an NLS into the D_2 receptor (D_2-nls), this did not alter the binding properties, with preserved agonist-detected high affinity and low affinity states, indicative of intact receptor-G protein coupling [7].

We expressed D_5 and D_2-nls dopamine receptors in cells and demonstrated heteromer formation, Fig. 2, since the D_2-nls receptor was able to translocate the D_5 receptor to the nucleus. Despite the lack of homology in the extensive c-tails of D_1 and D_5, in the D_5 receptor c-tail there are three adjacent glutamic acids (429-EEE) in a region comparable with the glutamic acid pair (404-EE) located in the D_1 receptor, Fig. 1. The D_1 receptor glutamic acids (404-EE) were identified as forming hetromers with the D_2 receptor [8]. We wished to determine if these three glutamic acids (429-EEE) located in the c-tail of the D_5 receptor were involved in forming heteromers with the D_2 receptor.

We prepared a series of six substitution constructs in the D_5 receptor c-tail (Table 1A), and each construct was expressed with the D_2-nls receptor. These D5 receptors expressed alone were located predominantly in the cytoplasm. The D_5 receptor c-tail constructs C1 (429-EEE to AAA), C2 (429-EEE to EAA) and C3 (429-EEE to EAE) all failed to show D_5–D_2 heteromerization (Fig. 2 and Table 1A), since the D_2 receptor did not translocate these D_5 receptors to the nucleus. D_5–D_2 heteromer formation was observed with D_5 receptor constructs C4 (429-EEE to AEE) and C5 (429-EEE to EEA), in which each contained the vicinal – EE residues. Also in C6 construct substitution of the adjacent aspartic acid (DEEE to AEEE) did not affect D_5–D_2 heteromer formation (Fig. 2 and Table 1A). These experiments indicated a heteromer requirement of at least a pair of glutamic acids (–EE) in the D_5 receptor c-tail. Thus like D_1 receptor it appears that in the D_5 receptor the equivalently located glutamic acid pairs were also involved in heteromerization of D_5–D_2 receptors. The presence of three glutamic acids (429-EEE) in the D_5 receptor, compared to two glutamic acids in (404-EE) in the D_1 receptor, would potentially allow two positions for oligomer formation with D_2 receptor, utilizing 429-EE or 430-EE.

3.2. Identification of the D_2 dopamine receptor amino acids involved in D_5–D_2 receptor heteromer formation

We wished to determine if the arginines (274–RR) located in ic3 of the D_2 receptor were involved in forming heteromers with the D_5 receptor. These arginines, identified as being involved in D_1–D_2 heteromers, were located a distance of 59 amino acids from transmembrane 5 (TM5), Fig. 1. The D_2-nls receptor with these arginines substituted (274–RR to AA) and the D_5 receptor were co-expressed, Fig. 2. These receptors D_5 and D_2-nls (RR to AA) did not form heteromers, confirming that both D_1 and D_5 dopamine receptors utilized the same residues in the dopamine D_2 receptor ic3 for heteromer formation.

3.3. Formation of D_5–D_1 dopamine receptor heteromers

The D_1-nls and D_5 receptors were co-expressed and formed heteromers, . 2, since the D_5 receptor was visualized translocating

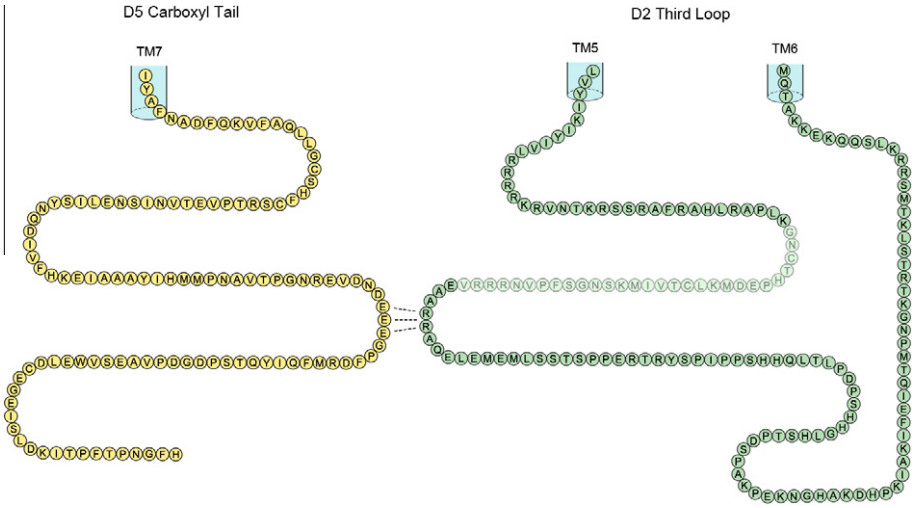

Fig. 1. Representation of the cytoplasmic intracellular tail of the D_5 dopamine receptor and the cytoplasmic intracellular third loop of the D_2 dopamine receptor. The position of the insert of 29 amino acids in the D_2 long receptor is indicated by shading.