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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

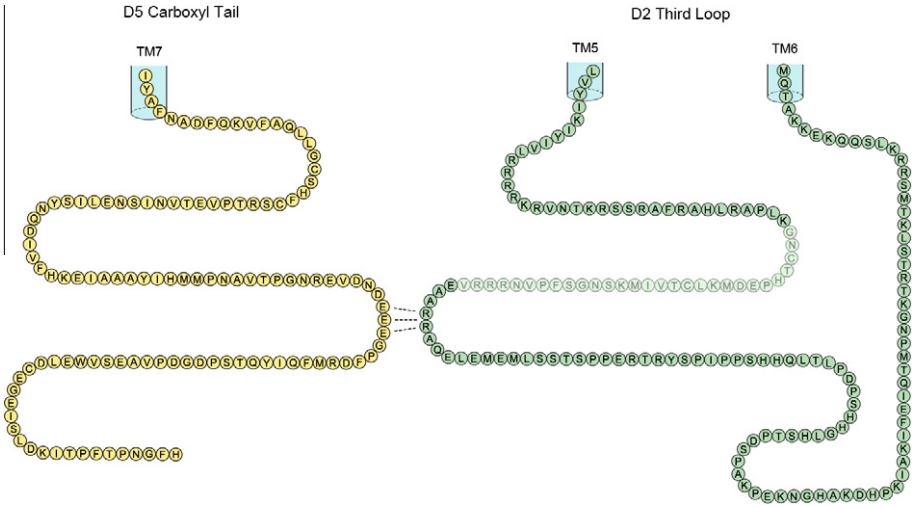

Fig. 1. Representation of the cytoplasmic intracellular tail of the D_5 dopamine receptor and the cytoplasmic intracellular third loop of the D_2 dopamine receptor. The position of the insert of 29 amino acids in the D_2 long receptor is indicated by shading.

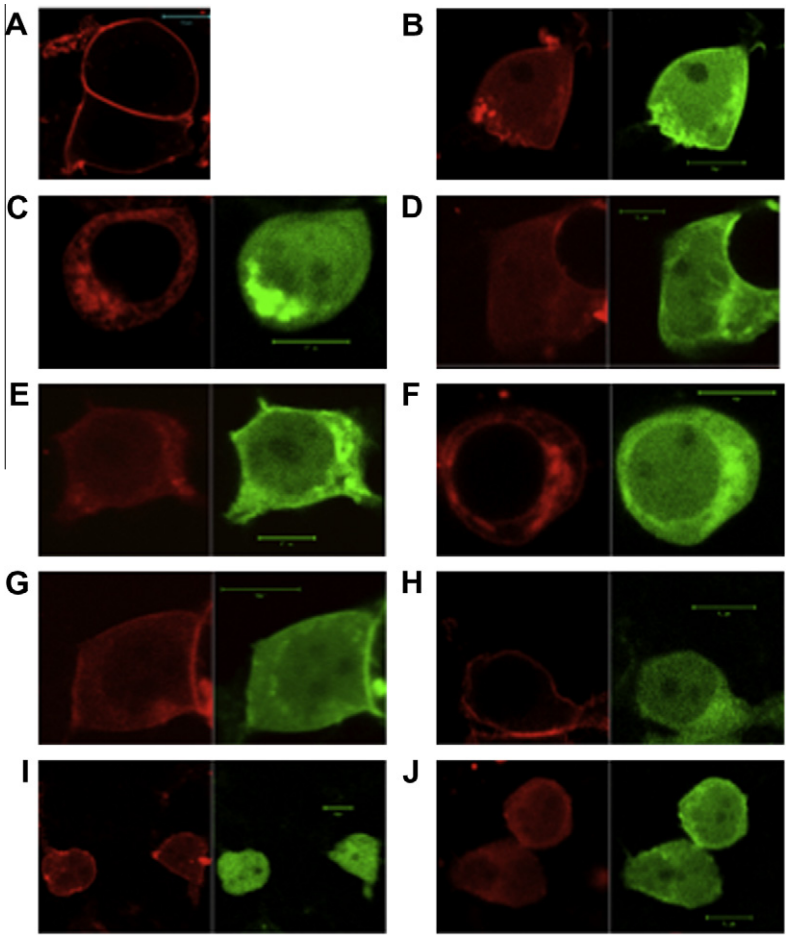

Fig. 2. Visualization of expression of D_5 and D_2-NLS dopamine receptors. (A) D_5 (RFP) (red), expressed at the cell surface. (B) D_5 (RFP) (red) and D_2-nls (GFP) (green) co-translocated to the nucleus. (C) C1 (D_5)(RFP) (red) and D_2-nls (GFP) (green) did not co-translocate to the nucleus. (D) C4 (D_5)(RFP) (red) and D_2-nls (GFP) (green) co-translocated to the nucleus. (E) C5 (D_5)(RFP) (red) and D_2-nls (GFP) (green) co-translocated to the nucleus. (F) C3 (D_5)(RFP) (red) and D_2-nls (GFP) (green) did not co-translocate to the nucleus. (G) C6 (D_5)(RFP) (red) and D_2-nls (GFP) (green) co-translocated to the nucleus. (H) D_5 (RFP) (red) and D_2-nls (RR to AA) (GFP) (green) did not co-translocate to the nucleus. (I) D_5 (RFP) (red) and D_1-nls (GFP) (green) co-translocated to the nucleus. (J) D_5 (RFP) (red) and D_1-nls (GFP) (green) co-translocated to the nucleus. Each size bar in figures showing cells indicates length of 10 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1A
D_5 dopamine receptor constructs.

		Heteromer
W/T	E V D N D E E E G P F D R	Yes
C1	E V D N D **A A A** G P F D R	No
C2	E V D N D E **A A** G P F D R	No
C3	E V D N D E **A** E G P F D R	No
C4	E V D N D **A** E E G P F D R	Yes
C5	E V D N D E E **A** G P F D R	Yes
C6	E V D N **A E E** E G P F D R	Yes

with D_1-nls to the nucleus. When D_1 receptor-nls was co-expressed with D_5 receptor construct, C1 (EEE to AAA), the D_1–D_5 heteromers remained together indicating that these D_5 receptor glutamic acids residues were not involved in forming D_1–D_5 heteromers.

4. Discussion

There are several accomplishments regarding the oligomeric structures of the D_5–D_2 dopamine receptors reported. (i) We determined that of three adjacent glutamic acids (429-EEE) in the c-tail of the D_5 receptor, any –EE pair was sufficient to form heteromers with the D_2 receptor. (ii) We determined adjacent arginines (274-RR) located in ic3 of the D_2 receptor, were involved in forming heteromers with the D_5 receptor. (iii) We identified single amino acid changes in the D_5 receptor that disrupted the D_5–D_2 heteromers. (iv) We also determined that in the c-tail of the D_5 receptor glutamic acid residues (429-EEE) were not involved in D_5–D_1 receptor heteromer formation. Thus our GPCR-nls incorporation strategy has now enabled elucidation of cytoplasmic structural features of two dopamine receptor families D_5–D_2, and D_1–D_2 receptor heteromers [8].

Despite the overall lack of homology in the ~ninety amino acids residues in the c-tails of D_1 and D_5 receptors, contiguous glutamic acids were located in equivalent positions and each shown to be involved in forming heteromers. The D_1 and D_2 dopamine receptors form heteromers with D_5 receptor using different interacting residues. Although the residues involved in D_1–D_5 heteromers have not yet been identified, potentially involving TM regions.

Interestingly the rat D_5 receptor c-tail contains five contiguous glutamic acids, presumably involved in forming heteromers with the D_2 receptor (Table 1B). Thus heteromer formation with these various glycine pairs, (potentially forming heteromers with any of four possible –EE pairs), interact with arginines in ic3 of the D_2 dopamine receptor permitting minor variations in the

Table 1B
D_5 dopamine receptor cytoplasmic tail sequence in mammalian species.

Human D5	E V D N D **E E E** G P F D
Rat D5	E V G **E E E E E** G P F D
Mouse D5	E V G **E E E E** A **E E E** G P F D
Dog D5	E V D K Q **E E** S P F D

conformation of the D_5–D_2 heteromer cytoplasmic structures. The mouse D_5 receptor has a total of four glutamic acids in a row then alanine and three additional contiguous glutamic acids, (Table 1B), allowing 6 different EE pairings in heteromer formation with the D_2 receptor. In comparison the D_1 dopamine receptor in human and rodent contains only a single glycine pair (404-EE).

Previously using the GPCR-nls strategy we identified discrete cytoplasmic regions in the mu and delta opioid receptors required for oligomer formation. In the carboxyl tail of the delta receptor we identified three glycine residues (-GGG), substitution of any of these residues prevented heteromer formation. In ic3 of both mu and delta receptors we identified three residues (-SVR), substitution of any of these residues prevented heteromer formation.

Thus data from our studies of four heteromer families, dopamine heteromers D_5–D_2, D_1–D_2, D_1–D_5, and mu-delta heteromers, indicate there is not a common mechanism for heteromer formation within Family A GPCRs. Receptor heteromerization arose in the cell to increase complexity, to develop and expand the utility and versatile range of functions of each individual GPCR, thus ways of interacting with other distantly related GPCRs as heteromers were opportunistically adapted. Whereas with closely related GPCRs such as D_1 or D_5, or mu and delta opioid receptors, these receptors maintained the formations used by homooligomer pairs, in forming heteromers.

In summary, we elucidated precise aspects of the cytoplasmic structure of D_5–D_2 receptor heteromers. By changing single amino acids in the D_5 receptor c-tail we succeeded is disrupting the D_5 receptor ability to form heteromers. We can now prepare D_5 and D_2 receptor expressing cells incapable of forming heteromers. Thus our work on the dopamine and opioid receptors is revealing the nature of the interactions involved in heteromers in the Family A GPCRs.

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