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Disruption of the mu–delta opioid receptor heteromer

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ABSTRACT

The crystal structure of the mu and kappa opioid receptors has revealed dimeric structural arrangements. Mu–delta receptors heteromers also exist and we have identified discrete cytoplasmic regions in each receptor 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 intracellular loop 3 of both mu and delta receptors we identified three residues (-SVR), substitution of any of these residues prevented heteromer formation.

1. Introduction

The recently reported crystal structures of mu and kappa opioid receptors described the oligomeric arrangement of these receptors as pairwise association of monomers [1,2]. Many G protein coupled receptors (GPCRs) form heteromers [3,4] and our goal is understanding how these receptors interact and the physiological relevance of heteromers. We, and others, have reported mu and delta opioid receptors also exist as heteromers [5,6], and these receptor interactions generated novel pharmacology and functional properties (recently reviewed [7]). Thus mu–delta heteromers provide an additional drug target with possible relevance in analgesia, tolerance and drug dependence.

Mu and delta receptors share 65% overall amino acid homology with 82% homology in the transmembrane domains, 87% in intracellular loop 3 but only 17% in the cytoplasmic tail. Previously, we partially identified a structural region required for the mu–delta interaction, which was present in the distal portion of the carboxyl tail of the delta receptor [8]. A delta receptor 15 amino acid carboxyl tail truncation had reduced ability to co-immunoprecipitate the mu receptor and definitive binding data indicated a lack of heteromer formation. Further structural details of the interaction sites of mu and delta receptor heteromers remained unknown.

In our ongoing investigations of receptor heteromer interactions we used a nuclear localization sequence (NLS) strategy; whereby an NLS was inserted into one of the receptors [9,10]. In previous investigations, the D2 dopamine–NLS receptor translocated the D1 dopamine receptor to the nucleus and thus provided a tool to study receptor:receptor dynamic interactions. By this means we successfully determined the structural basis for the D1–D2 dopamine receptor interaction. The precise contributions of cytoplasmic regions of these dopamine receptors to heteromer formation were identified [11].

In this report we determined the amino acids in the cytoplasmic regions of both delta and mu opioid receptors involved in heteromer formation. By changing a single identified amino acid in ic3 of mu or delta receptor or the carboxyl tail of delta receptor we prevented mu–delta heteromers from forming.

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 [9,11].

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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 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 of rat origin. Sequences encoding GPCRs were cloned into plasmids pEGFP, as described previously [11].

2.5. Receptor constructs

The mu and delta receptors were prepared using the Quickchange mutagenesis kit (Stratagene) according to the manufacturer's instructions, and as described [9,10]. Receptor DNA was subjected to PCR as previously reported [9,10]. 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 NLS sequence was inserted into DNA encoding the mu opioid receptor by PCR [12].

3. Results

3.1. Expression of the mu–NLS receptor with the delta receptor

The mu receptor expressed in HEK cells was localized on the cell surface, while expression of mu–NLS receptor revealed localization in cytoplasm and nucleus, Fig. S1A. Cells expressing mu–NLS were treated with naloxone (100 nM), resulting in cell surface retention and no receptor in the nucleus, Fig. S1B. Following naloxone removal, the distribution of the receptor was altered, as the mu–NLS receptor translocated mainly to the cytoplasm and nucleus, Fig. S1C. Heteromer formation was monitored by the ability of mu–NLS receptors to transport the delta receptor to the nucleus, the mu–NLS receptor and the delta receptor are shown co-expressed in Fig. 1A, and also following antagonist treatment and removal, Fig. 1B. In each case co-translocation of the mu and delta receptors was observed, indicating formation of mu and delta receptor heteromers.

3.2. Identification of carboxyl tail delta receptor residues involved in heteromer formation

As we had previously identified the distal carboxyl tail of the delta receptor as being involved in heteromer formation [8] we wished to identify specific residues involved. The carboxyl tail extends ~52 amino acids, comprising 14% of the total receptor, Fig. 2. We prepared a 15 amino acid carboxyl tail deletion construct (C1), and this was expressed with the mu–NLS receptor. This delta receptor, C1, failed to show receptor heteromerization, thus confirming critical amino acids in this region were required in heteromer formation. Deletion of the terminal 6 amino acids from this carboxyl tail (-GGGAAA), C2, resulted in no heteromer formation, this region is located 46 amino acids from the end of TM7. The terminal -AAA sequence was substituted (AAA to LLL), C3, and normal heteromer formation was observed. Whereas, substitution of three glycines with leucines, (C4, -*LLL*AAA) resulted in no heteromer formation, Fig. S1D. Thus these contiguous glycine residues were critical for delta interaction with the mu receptor. We prepared various additional constructs (Table 1) examining the role of each glycine, including C5 (AAG), C6 (GAG) Fig. 1C, C7 (AGG), and C8 (GGL) in each case no heteromers were formed. Also C12 and C13 (Table 1) we investigated the role of residues amino terminal to -GGG, with no effect on heteromer formation. Thus we identified three distal carboxyl tail residues (-GGG) and deletion of each residue prevented mu–delta heteromer formation.

3.3. Identification of mu opioid receptor residues involved in heteromer formation

To determine mu receptor residues involved in forming heteromers we prepared four constructs with deletions in the mu receptor carboxyl tail (C1–C4 in Table 2). This mu receptor carboxyl tail is 59 amino acids in length and comprises 15% of the receptor. Expression of each construct with delta receptor retained heteromer formation, thus eliminating 45 amino acids in the mu receptor carboxyl tail from direct involvement in heteromer formation.

We prepared a series of mu receptor constructs with deletions in ic3, this loop contains only ~23 amino acids, and comprises 6% of the receptor, Fig. 2. Each of the ic3 receptor constructs were expressed with the delta receptor. A deletion of 10 amino acids (L1, Table 2), from the mid-section of ic3 had no effect on mu–delta heteromer formation. A mu receptor with 3 substituted amino acids from the carboxyl region of ic3 (L2), also formed heteromers. However substitution of three residues, -SVR, from the amino terminal region of ic3, (L3), failed to form heteromers (Fig. 1D). Thus amino acids maintaining mu–delta receptor heteromer formation were contained in this discrete -SVR region. This ic3 sequence was located 4 amino acids from TM5 (Fig. 2). By substituting each of the three amino acids in this sequence, L4 (-<u>A</u>VR), and L5 (S<u>A</u>R), L6 (SV<u>A</u>) we determined that substitution of any of the residues in -SVR resulted in heteromer disruption. In the adjacent amino terminal sequence -RLK *SVR*, we substituted -R<u>A</u>K (L12) and -RL<u>R</u> (L11), each construct formed heteromers. A mu receptor with a conserved substitution of the serine with threonine -<u>T</u>VR, L10, did not form heteromers, also a conserved substitution of lysine for arginine (in -SV<u>R</u>) did not form heteromers, L8. Thus from a total of ~23 amino acids in ic3 of the mu receptor three amino acids were identified to be involved in forming heteromers. Thus we demonstrated a single residue change in mu ic3 prevented mu–delta heteromer formation.

3.4. Identification of ic3 delta opioid receptor residues involved in heteromers

The ic3 of delta receptor is 87% identical with mu, delta ic3 also contains the -SVR sequence, Fig. 2, in the similar location. We prepared a delta receptor with a substitution -SVR to -AAA in ic3 (Table 1), this delta receptor (L1) was expressed with mu receptor and failed to form heteromers, Fig. 1E.

4. Discussion

There are significant accomplishments regarding mu-delta receptor heteromer interactions reported. (i) We identified three contiguous glycines, -GGG, located in the delta receptor carboxyl tail required to form heteromers. (ii) We determined that each gly-

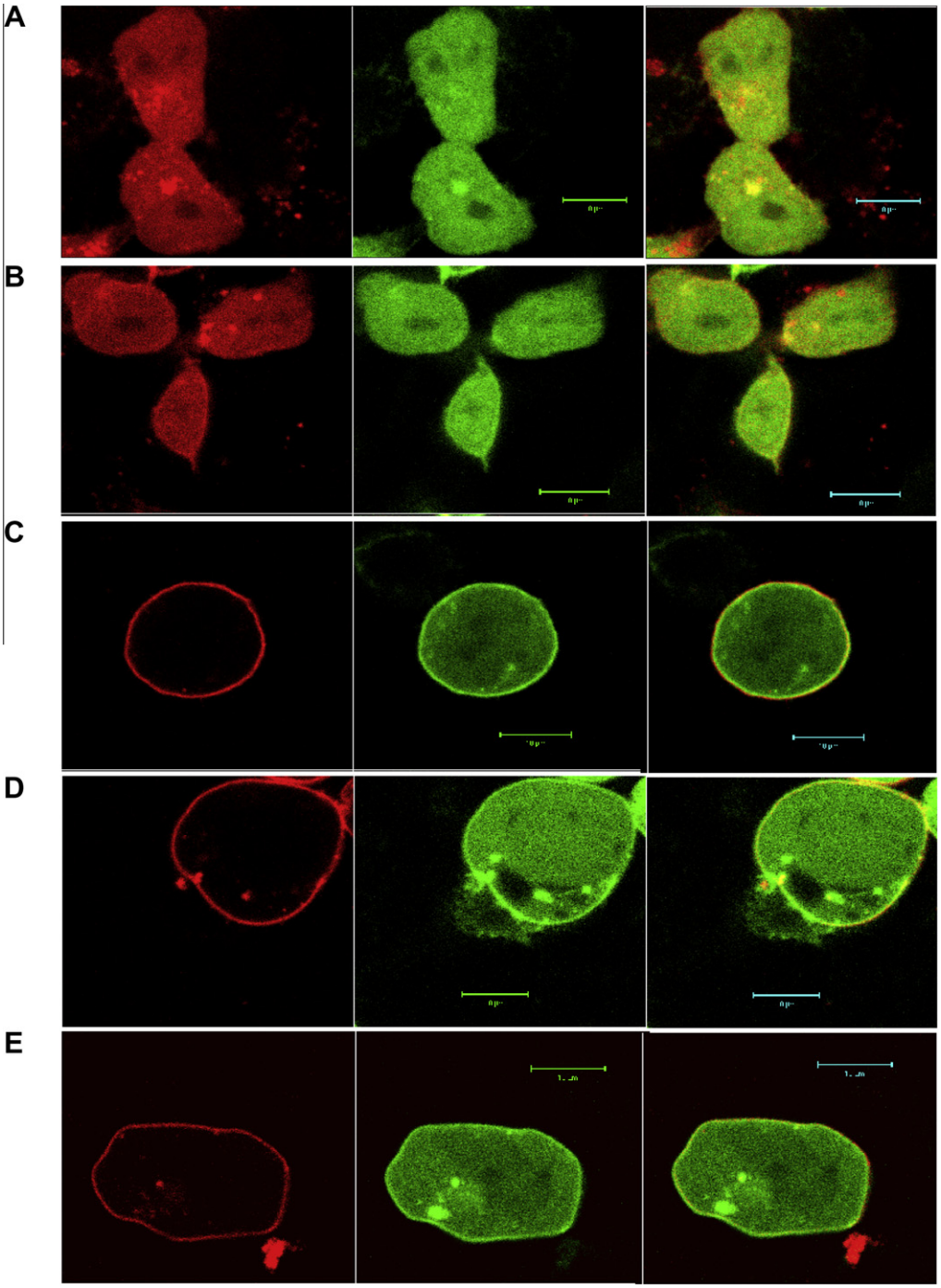

Fig. 1. Translocation of hetero-oligomers of mu and delta opioid receptors. (A). Mu–NLS (GFP) (green) and delta (RFP) (red) co-translocated to the cytoplasm and nucleus. The overlap in distribution of the two receptors is indicated by the merged image. (B). Mu–NLS (GFP) (green) and delta (RFP) (red) were co-expressed, treated with naloxone (100 nM), the receptors visualized following removal of the antagonist, the receptors migrated from the cell surface to the nucleus. (C). Mu–NLS (GFP) (green) and C6 delta (RFP) (red) were co-expressed, treated with naloxone (100 nM), the receptors were visualized following removal of the antagonist, only mu–NLS (GFP) (green) migrated from the cell surface to the nucleus. (D). L3 mu–NLS (GFP) (green) and delta (RFP) (red) were co-expressed, treated with naloxone (100 nM), the receptors visualized following removal of the antagonist, only L3 mu–NLS (GFP) (green) migrated from the cell surface to the nucleus. E. Mu–NLS (GFP) (green) and L1 delta (RFP) (red) were co-expressed, treated with naloxone (100 nM), the receptors visualized following removal of the antagonist, only mu–NLS (GFP) (green) migrated from the cell surface to the nucleus.

cine was involved, a delta receptor with any glycine substituted did not form heteromers. (iii) In mu ic3 we identified a contiguous sequence of three residues, -SVR, involved in forming heteromers. (iv) We determined that each of the mu receptor residues, -SVR, were required to form heteromers. (v) In delta receptor ic3 we determined an identically located -SVR sequence was involved in forming heteromers.

The receptor-NLS method has enabled precise elucidation of interacting structural features of GPCR heteromers. Previously we showed D1 and D2 dopamine heteromers interacted by electrostatic force involving two arginine residues in ic3 of D2 receptor and two glutamic residues in the carboxyl tail of the D1 receptor. Other methods attempting to identify specific residues involved in heteromer interaction such as resonance energy transfer method

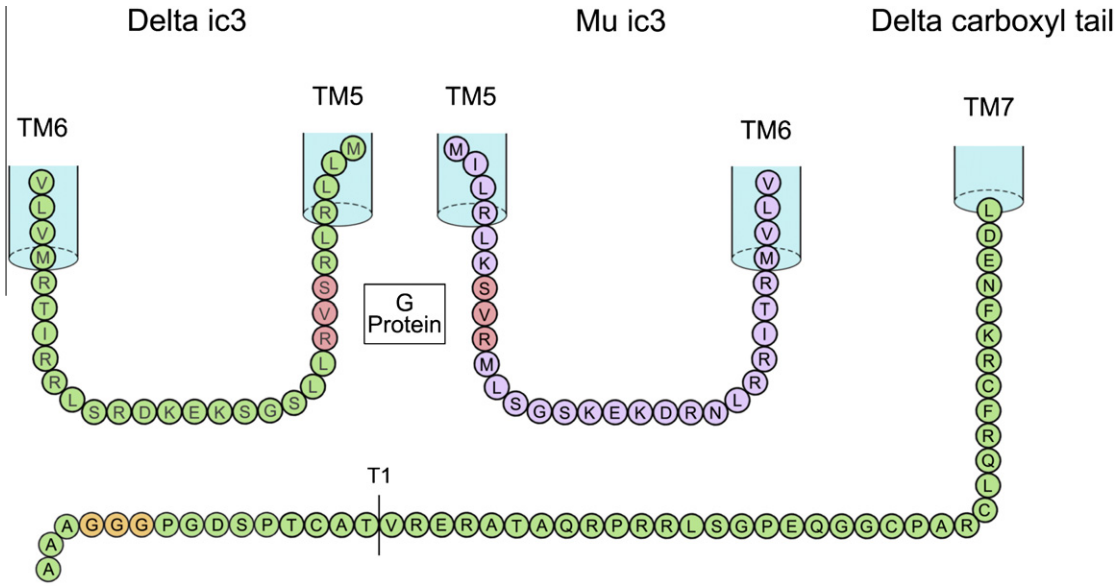

Fig. 2. Representation of the sequence of the carboxyl tail of the delta receptor and the intracellular third loop of the delta and mu receptor. The locations of the sites identified (-GGG) in the delta carboxyl tail and (-SVR) in ic3 of mu and delta are shown. T1 indicates the site of the delta carboxyl tail truncation [8].

Table 1
Underlined no bracket indicates substitutions.

Delta receptor constructs		Heteromer formation
W/TRVTACTPSDGPGGGAAA		
C1	RV(*T A C T P S D G P G G G A A A*)	No
C2	RV T A C T P S D G P (*G G G A A A*)	No
C3	RV T A C T P S D G P G G G *L L L*	Yes
C4	RV T A C T P S D G P *L L L* A A A	No
C5	RV T A C T P S D G P *A A* G A A A	No
C6	RV T A C T P S D G P G *A* G A A A	No
C7	RV T A C T P S D G P A G G A A A	No
C8	RV T A C T P S D G P G G *L* A A A	No
C9	RV T A C T P S D G P *L L L* (*A A A*)	No
C10	RV T A C T P S D G P G *L L* A A A	No
C11	RV T A C T *A* S *A* G *A* G G G A A A	Yes
C12	RV *A A A A A* S *A* G *A* G G G A A A	Yes
C13	RV *A A A A A* S *A* G *A* G G G A A A	Yes
W/T RLR SVR LLSG		
L1	RLR A A A LLSGS	No
L2	RLR *T*VR LLSGS	No

Table 2
Underlined no bracket indicates substitutions.

Mu receptor constructs		Heteromer formation
Wild type: CIPTSSTIEQQNSTRVRQNTREHPSTANTVDRTNHQLENLEATAPLP		
C1	DRTNHQ (*LENLEAETAPLP*)	Yes
C2	TREHPS (*TANTVDRTNHQLENLEATAPLP*)	Yes
C3	QNSTRV (*RQNTREHPS*) TANTV	Yes
C4	IP (*TSSTIEQQNSTR*) VRQNT	Yes
L1	RLKSVR (*MLSGSKEKDR*) NLRRI	Yes
L2	RLKSVRMLSGSKEKDR *A A A* RI	Yes
L3	RLK *A A A* MLSGS	No
L4	RLK *A* V R MLSGS	No
L5	RLK S *A* R MLSGS	No
L6	RLK S V *A* MLSGS	No
L7	RLK *S A A* MLSGS	No
L8	RLK S V *K* MLSGS	No
L9	RLK S *L* R MLSGS	No
L10	RLK *T* V R MLSGS	No
L11	RL *R* S V R MLSGS	Yes
L12	R *A* K S V R MLSGS	Yes

(BRET or FRET) have been less successful. BRET analysis reports frequently describe 'reductions' in a BRET signal but not separation of the receptor pairs, with failure to identify precise residues. Thus only the GPCR–NLS method can be applied to elucidate precisely the heteromer residues involved in this family of receptors.

Chaipatikul et al. [13] deleted the mu receptor ic3 sequence -RLK*SV*; this sequence contained -SV of -SVR. They reported this mu receptor could not activate G proteins treated with DAMGO, and also these mutant receptors did not interact with a co-expressed delta receptor. Also the ic3 delta receptor -RLR*S* deletion construct (this sequence containing S of SVR) failed to interact with the mu receptor. These investigators argued that mu–delta heteromer formation required the receptors to interact with G proteins [13]. Others determined that a 12 amino acid peptide derived from the proximal region of the mu receptor ic3 loop, which included the -SVR sequence interfered with coupling with G proteins [14]. This ic3 region proximal to TM5 has been implicated in G protein coupling in other GPCRs [15]. Other peptide receptor GPCRs such as somatostatin receptor SSTR2, dimerization required the presence of a G protein: Although, SSTR2 and SSTR5 heteromerization has been reported to require uncoupling from the G protein [16]. Also with neuropeptide Y–Y1 heteromers, treatment with pertussis toxin reduced neuropeptide Y–Y1 dimer population [17].

Thus many questions remain unresolved by the findings presented herein. How the identified delta receptor residues in carboxyl tail, -GGG, participate in the oligomer mechanism is not known. In the delta receptors construct (-GGG to AAA) G protein coupling was observed, but not heteromer formation (unpublished data). Neither is the role of the -SVR ic3 structure understood in both mu and delta opioid receptors, although the -SVR sequence may involve G protein interactions. Also unknown is why the delta receptor requires cytolasmic sequences in both ic3 and carboxyl tail involved in heteromer formation. It is noteworthy that the -SVR sequence is also present in the same ic3 location in the kappa opioid receptor. The presence of -SVR sequence in mu, delta and kappa sequence indicates that the opioid receptors likely form het-

eromers through identical mechanisms involving ic3. Given the high homology of these receptors it is possible that the same regions involved in heteromer are also involved in homo-oligomer formation. Still other receptor regions involved in oligomer formation likely will be identified, perhaps in the TM regions [1].

Thus data from our studies of two heteromer families, D1–D2 dopamine and mu–delta heteromers, indicate there is not a common mechanism for heteromer formation within family A GPCRs. A common mechanism could be expected with closely related receptors, such as the three opioid receptors, where ic3 and carboxyl tail are similar in size in each receptor. However the D1 and D2 dopamine receptors are more distantly related, thus D1 and D2 dopamine receptors have evolved a different method for heteromer formation.

Heteromer formation for mu–delta opioid receptors modifies the cytoplasmic environment, and these changes in intracellular regions enable participation in G protein coupling of different signaling cascades, mu–delta heteromers showed higher affinities of interaction for Gz over Gi [18].

In summary we successfully elucidated several cytoplasmic regions involved in mu–delta receptor heteromers, it is now possible to prepare mu–delta receptor expressing cells engineered to be incapable of forming heteromers. The properties of such cell lines will be of interest to further elucidate roles of GPCR heteromers in physiology.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.05.023.

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