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Expression of the third Intracellular Loop of The Δ-Opioid Receptor Alters Opioid Receptors' Signaling

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INTRODUCTION-AIM

Opioid receptors belong to the large superfamily of G protein coupled receptors (GPCRs) and modulate a variety of physiological responses in the nervous system, via coupling to multiple patterns of G proteins. All three opioid receptors (μ,δ,κ) share a high degree of homology, particularly between the intracellular loops, known to be involved in G protein coupling and activation (1 and references therein). Previous results from our laboratory concerning the interactions of opioid receptors with G proteins have shown that the third intracellular loop of the δ-opioid receptor is responsible for G protein coupling and inhibition of adenylyl cyclase (2-4). Based on these observations and in an attempt to design and develop analogues that could potentially block or activate selectively in vivo, the interactions of these receptors with G proteins or other downstream signaling components, we constructed a minigene encoding the third intracellular loop of the murine δ-opioid receptor (pDORi3). Minigene plasmid vectors are constructs designed to express relatively short polypeptide sequences, following by their transfection into mammalian cells (5). Our results indicate that the pDORi3 minigene is able to interact specifically with the G proteins in HEK293 cells expressing stably the μ-opioid receptor. These observations underlie the significance of the third intracellular loop for signal transfer of opioid receptors, and suggest a strategy whereas the receptor-G protein interface may represent a target for novel receptor drug development.

METHODS

Construction of the pDORi3 minigene: Two oligonucleotides that were partly complementary encoding the 23 amino acids of the third intracellular loop of murine δ-opioid receptor were synthesized and were allowed to anneal. After digestion with restriction enzymes the cDNA was ligated to the eukaryotic plasmid vector pcDNA3. The presence of the insert was verified by automated dideoxynucleotide sequencing (ABI Prism 377 DNA Sequencer).

Cell culture and transient transfections: Human embryonic kidney, HEK293 cells stably expressing the μ-opioid receptor (μ-HEK293) were used. Membranes from these cells were prepared as described in (2). Transient transfections were carried out using lipofectamine according to the manufacturer's instructions (GIBCO BRL).

Receptor binding: Receptor binding was performed at 30 °C for 45 min in buffer containing 20 mM Tris-HCl, pH 7.5, 50mM sucrose and 20mM $MgCl_2$ as described in (3).

Measurements of cAMP accumulation: μ-HEK 293 cells, were transiently transfected with empty vector pcDNA3 or pDORi3 minigene, and cultured in 6 well plates for 72 h. Cells were incubated with various concentrations of DAMGO ranging from 10nM-10μM and 10μM forskolin for

30 min in 37°C, and the cAMP content of the supernatants was determined according to Megaritis et al (4).

[^{35}S]GTPγS binding: [^{35}S]GTPγS binding was performed as described previously in (3).

RESULTS AND DISCUSSION

To determine whether we could selectively antagonize G protein signaling mechanisms in intact cells by expressing peptides that interact with the receptor-G protein interface, we generated a minigene construct that encodes the third intracellular loop of the δ-opioid receptor (pDORi3) and successfully expressed it transiently in HEK293 cells. To examine whether the presence of the third intracellular loop encoded by the pDORi3 minigene would be able to modify opioid binding affinity of μ-HEK293 cells, specific [^{3}H]diprenorphine (antagonist) and [^{3}H] DAMGO (agonist) binding was tested. For that reason, whole μ-HEK293 cells transiently transfected either with pcDNA3 or pDORi3 minigene were used for a scatchard analysis with the above ligands. As shown in Table 1, the presence of pDORi3 did not alter the binding characteristics of [^{3}H]diprenorphine. In contrast, when [^{3}H] DAMGO was used, the presence of pDORi3 resulted in a significant alteration both in Kd and Bmax values indicating that the minigene interferes in the effective coupling between the μ-opioid receptor and the G proteins.

To determine whether or not the expression of the pDORi3 minigene had any effect on μ-opioid receptor-mediated inhibition of adenylyl cyclase, similar cotransfections as described above were performed and subsequently the levels of cAMP accumulation were measured. As shown in Figure 1, the presence of pDORi3 resulted in a more profound reduction in cAMP accumulation than that observed with vector alone.

Table 1

Receptor type	Co-transfected plasmid	[^{3}H]diprenorphine binding		[^{3}H] DAMGO binding	
		*K*d (nM)	*B*max (pmol/mg)	*K*d (nM)	*B*max (pmol/mg)
μ-opioid	pcDNA3	1.95±0.7	4.74±1.4	2.25±0.3	5.25± 0.2
μ-opioid	pDORi3	2.34±0.8	5.34±1.3	1.44±0.1	2.2 ±0.2

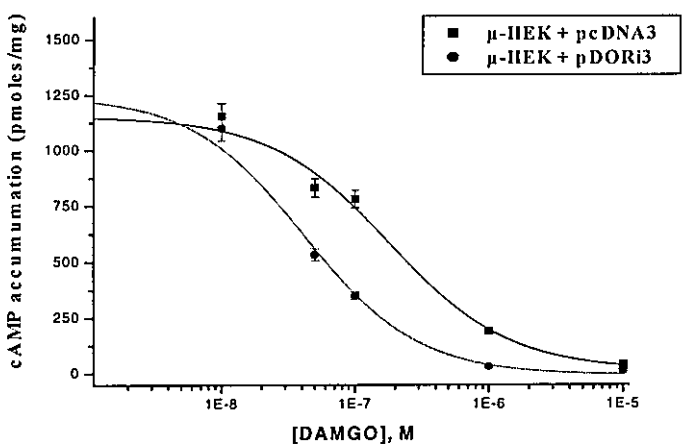

Figure 1: Effect of pDORi3 minigene on DAMGO induced inhibition of cAMP accumulation in μ-HEK293 cells. ■: μ-HEK+pcDNA3, •: μ-HEK+ pDORi3

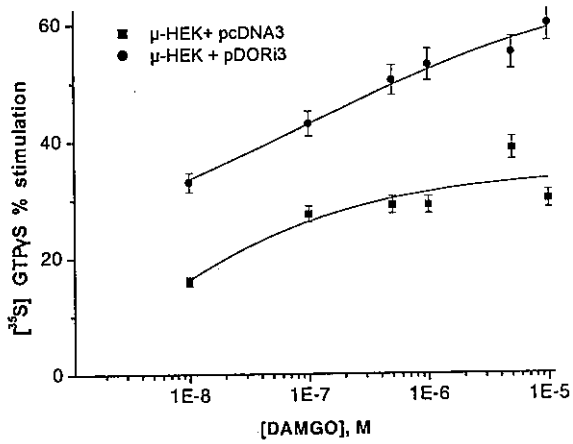

Figure 2: Effect of pDORi3 minigene on DAMGO induced stimulation of [^{35}S] GTPγS binding. ■: μ-HEK+pcDNA3, •: μ-HEK+ pDORi3

To provide additional evidence for the possible significance of third intracellular loop in the interactions of the μ-opioid receptor with the cellular G protein(s), we examined the ability of pDORi3 to modulate agonist stimulation of [^{35}S]GTPγS binding in μ-HEK293 cell membranes transfected either with pDORi3 or with pcDNA3. Figure 2, shows that DAMGO produced a consistent stimulation of [^{35}S]GTPγS binding which was increased in the presence of the minigene.

Taken together our data indicate, that cellular expression of the third intracellular loop of the δ-opioid receptor expressed by the pDORi3 minigene, interferes with the μ-opioid receptor signaling in intact cells, as it diminishes the DAMGO mediated cAMP accumulation Supplementary studies with DAMGO stimulation of [^{35}S]GTPγS binding indicated that the presence of the minigene results in effective G protein activation. The specificity by which the third intracellular loop peptide interferes in opioid receptor mediated signaling suggests the feasibility of developing drugs that might exert an inhibitory or stimulatory effect at the level of receptor-G protein interface rather than at the level of ligand-receptor binding.

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