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Application Note

Reporter Gene Assays for the Investigation of Signal Transduction at Orexin Receptors

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The Orexin Receptor System

In 1998 the research group of Yanagisawa isolated the neuropeptides orexin-A and orexin-B from rat brain extracts (1). These peptides are mainly produced by a neuron cluster in the lateral hypothalamus and bind to two G protein-coupled receptors, termed orexin receptor (OxR) 1 and 2 (1). For signal transduction both receptors couple to and activate G_q proteins, leading to production of inositol triphosphate species (IP_3) and an intracellular rise in Ca^{2+}, or to stimulation of the MAPK pathway (2). Furthermore, OxR2 is able to modulate the intracellular concentration of cAMP by coupling to G_s and G_i proteins (3). The orexin receptor system is involved in many physiological processes. In addition to an appetite-stimulating function (1), it has been shown that orexins and their receptors regulate the sleep/wake cycle (3). Thus, orexin receptors are highly interesting as pharmacological targets to treat sleep disorders. In this Application Note, we demonstrate use of reporter gene assays to investigate signal transduction of orexin receptors, and the activity and binding of OxR ligands.

Activation of OxR2 Signaling Pathways

We used a reporter gene assay system from Promega to investigate the signal transduction of OxR2. In this cell-based assay, a receptor plasmid and a pGL4 reporter plasmid carrying an inducible luciferase gene are cotransfected into mammalian cells. After ligand binding, the receptor is activated and by cellular signal transduction processes transcription factors are expressed. These transcription factors bind to promotor elements in the reporter plasmid and induce luciferase expression. The enzymatic activity of the luciferase was determined using the ONE-Glo™ Luciferase Assay System. We used the following reporter constructs to investigate the different OxR2 signaling pathways:

- cAMP pathway (pGL4.29, CRE reporter)
- IP_3/Ca^{2+} pathway (pGL4.30, NFAT reporter)
- MAPK pathway (pGL4.33, SRE reporter).

After transfection into HEK293 and CHO cells the newly expressed OxR2 receptors were stimulated with orexin-A and orexin-B. Dose-response curves were determined by luciferase activity (Figure 1A and 1B) and EC_{50} values were calculated The EC_{50} value is the ligand concentration at which 50% of the maximum receptor activity is reached. EC_{50} values were calculated from dose-response curves using GraphPad® Prism software version 5.03. Much higher orexin peptide concentrations were necessary to stimulate the cAMP pathway compared to IP_3/Ca^{2+} signaling and MAPK pathways (EC_{50}-values cAMP: 110-255 nM; EC_{50}-values IP_3/Ca^{2+} and MAPK: 0,2 – 3,6 nM). This result implies that the different cellular G proteins bind to the active conformation of the OxR2 with different efficiencies. The reporter gene assay is sensitive enough to detect weak signaling pathways, like the cAMP pathway/OxR2 interaction.

Identification of Amino Acid residues Critical for OxR2 Activation

For drug development it is crucial to understand which amino acids in the orexin receptor participate in receptor activation and ligand binding. Identification of these amino acids can be determined experimentally by genetically replacing individual amino acids in the orexin receptor with the „neutral“ amino acid alanine (A). The effect of the mutated amino acid on the receptor-ligand interaction can then be be investigated by comparison to the wild type receptor. To demonstrate that such orexin receptor mutants can be characterized by reporter gene assays, we replaced the amino acids isoleucine^{320} (I^{320}) and asparagine^{324} (N^{324}) in OxR2 with alanine. Both amino acids are located in regions of the OxR2 protein that are important for receptor activation. The mutated receptors were transfected into CHO cells and their activity investigated after orexin-B stimulation (Figure 2). The mutation $I^{320}A$ had no effect on signal transduction. The EC_{50}-values calculated from dose-response curves of the wild type OxR2 and the OxR2-$I^{320}A$ mutant were almost equal (1,2 nM for wt-OxR2 and 1,9 nM for OxR2-$I^{320}A$). Therefore, the amino acid I^{320} has little influence on receptor activation. In contrast, the EC_{50}-value of the OxR2-$N^{324}A$ mutant decreased nearly 40-fold compared to that of wt-OxR2 (1,2 nM for wt-OxR2; 40,9 nM for OxR2-$N^{324}A$). Such a dramatic difference suggests participation of the amino acid N^{324} in activation of OxR2. These data are consistent with the

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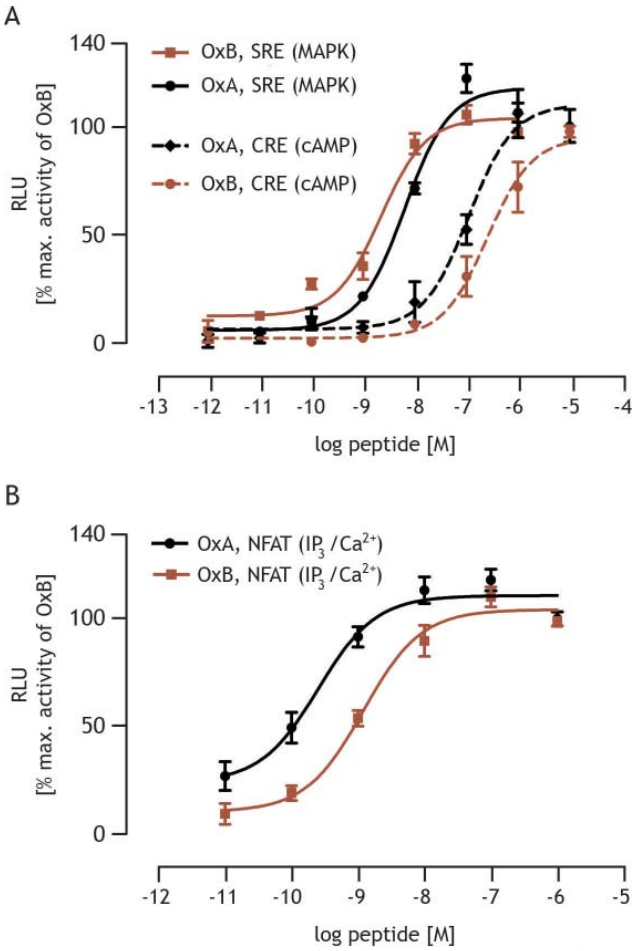

Figure 1. Dose-response curves for receptor activation of the OxR2 after stimulation with orexin-A and orexin-B. In Panel A the cAMP (CRE) and MAPK (SRE) pathway in HEK293 cells is stimulated and in Panel B the IP_3/Ca^{2+} (NFAT) signaling in CHO cells is activated.

results from a study that used a Ca^{2+}/FLIPR assay to investigate the same OxR2 mutants (4).

Conclusions

Reporter gene assays are a useful tool for the investigation and characterization of orexin receptor signaling. In this paper, the reporter assay provided a single test system covering the different signaling pathways of the orexin receptors. The reporter assay could also be used to investigate the influence of individual amino acids on the activation of orexin receptors. Knowledge of the amino acids that are involved in the ligand:receptor interaction is a crucial prerequisite to the development of small molecule compounds that target orexin receptors.

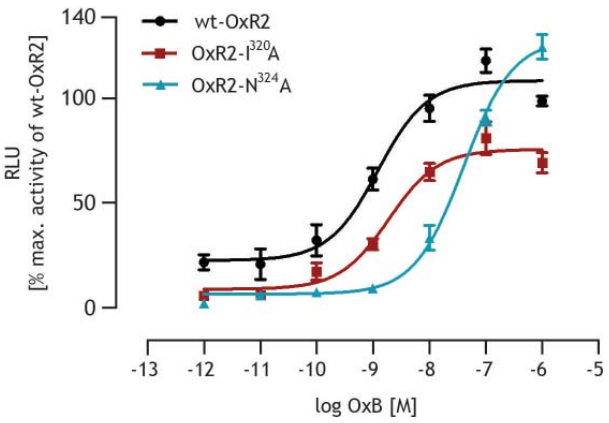

Figure 2. Dose-response curves of orexin receptor mutants after stimulation with orexin-B in CHO cells.

References

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