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CRH-R1α Structural Domains Critical for Gs-Protein Activation: Evidence for Distinct Receptor Active Conformations Associated with CRH and Urocortin Binding

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INTRODUCTION

CRH, the 41 amino acid hypothalamic peptide, plays a key role in the integration of the neuroendocrine, behavioural, autonomic and immune responses to stress. The primary role of CRH is to activate the hypothalamo-pituitary-adrenal (HPA) axis. The brain contains a diverse network of CRH-R and employs a variety of intracellular mechanisms for regulating CRH actions. Recently it has become clear that CRH is part of a larger family of stress related peptides, which include the mammalian urocortins as well as the frog peptide sauvagine and the fish peptide urotensin-I.

CRH related peptides mediate their effects on target cells via plasma membrane receptors, which belong to the family of the seven helical transmembrane domain receptors (GPCRs) and transduce their signal through stimulation of various G proteins. Two CRH receptor (CRH-R) subtypes have been isolated (CRH-R type-1, CRH-R1 and CRH-R type-2, CRH-R2) with distinct tissue distribution and pharmacology. Both subtypes are primarily coupled to the adenylyl cyclase system, although they are capable of activating multiple subtypes of G-proteins in a tissue-specific manner (1). The CRH-R1 binds CRH and urocortin (Ucn) with similar affinity but not Ucn II and III and is expressed as multiple variants with structural differences and different pattern of G-protein activation. Interestingly, although the splice variant alpha of CRH-R1 (CRH-R1α) binds CRH and urocortin with equal potency, the two peptides can activate G-proteins and signalling cascades with different potency in a tissue specific manner (2).

Therefore, in different target tissues the ability of CRH and CRH-like agonists to elicit a cellular response ultimately depends both on the ability of CRH-R to agonist-activate certain G-proteins and second messengers and on the intrinsic ability of these second messengers to trigger the necessary intracellular signalling pathways in the particular cellular system. This observation emphasises the importance of the complex agonist-receptor as the prime determinant of activation of a signalling cascade and places CRH receptor in a central role in *dictating* the intracellular responses of agonists, depending on the G-proteins it can activate. The structural basis for this is unknown.

METHODS

The cDNAs encoding wild type CRH-R1α was subcloned into pcDNA 3.1(-) (Invitrogen), thus creating the vector, CRH-R1α-pcDNA3.1(-). Mutations were constructed using an overlapping PCR mutagenesis strategy and as a template the CRH-R1α-pcDNA3.1(-) (3). The polymerase chain reaction-generated DNA fragments containing the mutations were subcloned into the CRH-R1α-pcDNA3.1(-) plasmid, and the mutations were confirmed by DNA sequencing.

Wild type CRH-R1a and all mutant receptors were transiently transfected into HEK 293 cells using the Lipofectamine method. cAMP stimulation assays, using HEK293 cells expressing wild type and mutant CRH-R1α receptors, were carried out as previously described (2). Cyclic AMP production was measured using a cAMP RIA kit (Biomedical Technologies Inc.).

RESULTS

In order to identify domains in the sequence of the splice variant alpha of CRH-R1 (CRH-R1α) that are important for Gs-protein coupling, we replaced with alanine (Ala) specific cassettes of amino acids located at the intracellular regions of receptor. We focused on the N- and C-terminus of the 3rd intracellular loop (IL3) of CRH-R1α since in most seven transmembrane domain G-protein coupled receptors (GPCRs), amino acids within the 3rd intracellular loop (especially the N- and the C-terminus of the loop) have been shown to be critical for efficient G-protein coupling and signal transduction.

Ala substitution of the amino acid cassette R^{292}-I^{293}-L^{294}-M^{295}, which is located at the N-terminus of IL3 of CRH-R1α augmented CRH-induced cAMP production by 2.5-3 fold without affecting basal receptor activity. In order to determine the role of each of the above amino acids on receptor/G-protein interaction we mutated them to Ala, one at a time. Single amino acid substitutions demonstrated that R^{292} plays a critical role in CRH-R1α-Gs-protein interaction. Similar results were obtained with CRH-R1α mutants containing Ala substitution of the amino acid cassette K^{311}-A^{312}-V^{313}-K^{314}, which is located at the C-terminus of the IL3 of CRH-R1α. Interestingly, these mutations had a lesser impact on Ucn induced adenylyl cyclase activation; Ala substitution of the amino acids in the N-terminus of IL3 increased Ucn-induced cAMP response 1-1.5 fold, whereas mutations at the C-terminus of IL3 did not alter Ucn-induced cAMP response.

In marked contrast, simultaneous Ala substitutions of the side chains of the amino acids in both IL3 regions totally abolished CRH- or UCN-induced cAMP response.

CONCLUSIONS

Both the N- and the C-terminus regions of IL3 of the CRH-R1 play an important role in the effective coupling of the CRH-stimulated active conformation of receptor to Gs-proteins. Simultaneous removal of the side chains from the amino acids in the N- and the C-terminus IL3 regions, by mutating them to Ala, totally abolished CRH-induced cAMP response. The important role of the N- and the C-terminus regions of IL3 of CRH-R1 in receptor/Gs-protein interaction is further supported by that each of these regions alone (by Ala substituting the amino acids in the other region) is sufficient for the receptor to elicit a response after its activation by agonists. Ala substitution of either the N-terminus or the C-terminus region of IL3 of CRH-R1a results in an increase of the response elicited by CRH stimulation of receptor. These results suggest that Ala substitution in these regions facilitates the interaction of the intracellular regions of CRH-R1α with Gs-proteins, thereby allowing more potent coupling and activation of Gs-protein. Such facilitation might be due to a conformation change of CRH-R1α that enriches the receptor into an active state, which more favourably interacts with Gs-proteins. Remarkably, in contrast to CRH, the Ucn-stimulated cAMP accumulation is enhanced by removing the side chains (with Ala substitution) from the amino acids located only at the N-terminal region of IL3 of CRH-R1α. Ala substitution of the amino acids in the C-terminus region of IL3 was without effect on Ucn-stimulated cAMP accumulation. This leads to the hypothesis that the active conformation of CRH-R1α associated with Ucn binding is most likely different than that associated with CRH binding. This is further supported by previous studies in which Ucn and CRH have been shown to stimulate different sets of G-proteins and different intracellular pathways (2)

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