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The Molecular Mechanisms Underlying the Regulation of the Biological Activity of Corticotropin-Releasing Hormone Receptors: Implications for Physiology and Pathophysiology

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The CRH receptor (CRH-R) is a member of the secretin family of G protein-coupled receptors. Wide expression of CRH-Rs in the central nervous system and periphery ensures that their cognate agonists, the family of CRH-like peptides, are capable of exerting a wide spectrum of actions that underpin their critical role in integrating the stress response and coordinating the activity of fundamental physiological functions, such as the regulation of the cardiovascular system, energy balance, and homeostasis. Two types of mammal CRH-R exist, CRH-R1 and CRH-R2, each with unique splicing patterns and remarkably distinct pharmacological properties, but similar signaling properties, probably reflecting their distinct and sometimes contrasting biological functions. The regulation of CRH-R expression and activity is not fully elucidated, and we only now begin to fully understand the impact on mammalian pathophysiology. The focus of this review is the current and evolving understanding of the molecular mechanisms controlling CRH-R biological activity and functional flexibility. This shows notable tissue-specific characteristics, highlighted by their ability to couple to distinct G proteins and activate tissue-specific signaling cascades. The type of activating agonist, receptor, and target cell appears to play a major role in determining the overall signaling and biological responses in health and disease. (*Endocrine Reviews* 27: 260–286, 2006)

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Abbreviations: CNS, Central nervous system; CREB, cAMP response element-binding; CRF, corticotropin-releasing factor; CRH-BP, CRH binding protein; CRH-R, CRH receptor(s); CT-R, calcitonin receptor; 3D, three-dimensional; EC, extracellular; ECD, EC domain; GPCR, G protein-coupled receptor; GRK, G protein-coupled receptor kinase; h, human; HPA, hypothalamo-pituitary-adrenal; h/rCRH, human/rat CRH; IC, intracellular; iNOS, indicible NOS; LD, linkage disequilibrium; o, ovine; NO, nitric oxide; NOS, NO synthase; PKA, protein kinase A; PKC, protein kinase C; PTH-R, PTH receptor; PVN, paraventricular nucleus; SCR, short consensus repeat; sGC, soluble guanylyl cyclase; TMD, transmembrane domain; UCN, urocortin; VMH, ventromedial hypothalamic.

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I. Introduction

STRESS IS AN ancient phenomenon that can be traced back through evolution. The concept of stress, however, is modern, and describes a complex set of adaptive physiological responses to external demand. Survival is therefore dependent upon an adequate response to stressful stimuli. This process involves activation of a number of different but integrated physiological responses involving the autonomic, endocrine, immune, cardiovascular, and reproductive systems, which induce a spectrum of behavioral and homeostatic changes. During evolution, mammals have evolved remarkably similar molecular signals that orchestrate the integrated stress response. The conservation of these molecules is a reflection of their role in survival and adaptation. In the mammalian adaptive response to stress, the hypothalamo-pituitary-adrenal (HPA) axis plays a central role, predominantly via the hypothalamic hormone, CRH or corticotropin-releasing factor (CRF), which regulates the secretion of adrenocorticotropin from the anterior pituitary. In addition, CRH exerts a wide spectrum of actions in the central nervous system (CNS) and the periphery that underpin

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its critical role in integrating and coordinating the activity of diverse physiological systems. Interestingly, this complex process of stress adaptation is fine-tuned by several CRH-related peptides, namely the urocortins (UCNs) that exert complementary or sometimes contrasting actions.

CRH and the UCNs are expressed in multiple sites in the periphery, where they influence physiological mechanisms via paracrine or autocrine activation of specific receptors expressed on the cell membrane of target cells. These CRH receptors (CRH-Rs) and the mechanisms regulating their activity play a crucial role in mediating the biological effects of the CRH family of peptides. This review will discuss current knowledge relating to the structure, biological function, and molecular mechanisms regulating CRH-R expression and activity.

II. CRH-R Agonists: the Family of CRH-Related Peptides

The first mammalian CRH peptide was isolated from ovine hypothalamic extracts in 1981 (1). This was followed by the discovery of a novel family of mammalian CRH-related ligands, which at present contains three members: UCN-I, UCN-II (or stresscopin-related peptide), and UCN-III (or stresscopin) (2–5). These peptides appear to stem from an ancestral peptide precursor (6).

CRH, a 41-amino acid peptide, is the principal regulator of the basal and stress-induced pituitary-adrenal axis that activates glucocorticoid and adrenal androgen secretion (7, 8). It displays anxiogenic properties and coordinates the adaptive, behavioral, and physical changes that occur during stress; this has been conclusively demonstrated in transgenic or knockout mouse models (9, 10). Some of these actions of CRH may be mediated via the activation of norepinephrine-secreting neurons and other neurotransmitter systems important for mood regulation (11–14). CRH also influences appetite via its anorexic properties (15, 16). In addition, CRH is synthesized and produced in multiple peripheral tissues and might be involved in many other biological functions, such as energy balance, metabolism, and regulation of the immune response (17, 18). Interestingly CRH also plays a role in mammalian reproduction and embryo implantation (19). It is synthesized and secreted by the human placenta and might act as a "placental clock" that regulates the onset of human labor, perhaps by modulating myometrial contractility (20, 21). Increased or chronic secretion of CRH leads to anxiety, sleep pattern disturbance, and changes in the cardiovascular, metabolic, and immune functions. CRH has been implicated in the pathophysiology of various disorders; in particular, there is good evidence linking CRH system abnormalities to chronic anxiety disorder, melancholic and atypical depression, chronic pain and fatigue states, sleep disorders, addictive behavior, neurodegeneration, allergic and autoimmune inflammatory disorders, the metabolic syndrome, and gastrointestinal diseases (22–27).

The UCNs exhibit various degrees of amino acid sequence homology to CRH: UCN-II shows moderate identity with human/rat CRH (h/rCRH) (34%), human UCN-I (43%), and UCN-III (37–40%), whereas UCN-III displays less homology to other members of the CRH family (18–32% identity). They are also expressed in the CNS and peripheral tissues; however, their actions are less well characterized. In the brain, UCN-I expression is most prominent in the Edinger-Westphal nucleus and the lateral superior olive. There is very little neuroanatomical overlap in the distribution of UCN-I and CRH in the brain, suggesting differential functional roles for these peptides (28). UCN-I mRNA or immunoreactivity has also been reported in other brain regions including the cerebellum, hippocampus, neocortex, olfactory system, basal ganglia, amygdala, and the supraoptic, ventromedial (VMH), and paraventricular (PVN) nuclei of the hypothalamus, as well as the laterodorsal tegmental nucleus, the dorsal raphe, the periaqueductal gray, the substantia nigra pars compacta, and ventral tegmental nucleus. In the latter two regions, UCN-I immunoreactivity is located almost exclusively in neurons that coexpress tyrosine hydroxylase, suggesting that within these regions, UCN-I is colocalized with dopamine. Interestingly, several brain regions such as the ventromedial hypothalamus and the amygdala (tissues rich in CRH-R2 receptors that exhibit significant selectivity for the UCNs; see *Section III.A*) do not contain UCN-I immunoreactive fibers. UCN-I expression has also been demonstrated in peripheral tissues such as the heart, adrenal, skeletal muscle, placenta, skin, immune system, and the gastrointestinal tract (29).

A similar pattern of distribution to that of UCN-I was found for UCN-II mRNA in the mouse and rat CNS (29). UCN-II mRNA is highly expressed in the paraventricular, supraoptic, and arcuate nuclei of the hypothalamus, the locus coeruleus, and motor nuclei of the brain stem and spinal cord. The posterior part of the bed nucleus of the stria terminalis, the lateral septum, and the medial amygdaloid nucleus are important brain sites that express UCN-II mRNA. Histologically, UCN-II mRNA and UCN-II-like immunoreactivity were demonstrated in both the anterior and intermediate lobes of the pituitary, but not detected in the posterior lobe. In the periphery, high levels of UCN-II mRNA have been detected in the heart, adrenal gland, placenta, stomach, skin, ovary, gastrointestinal tract, uterus, uterine smooth and skeletal muscle, and peripheral blood cells (29).

UCN-III displays a distinct distribution from that of CRH, UCN-I, and UCN-II (30). UCN-III-positive neurons were found predominately within the hypothalamus and medial amygdala. In the hypothalamus, UCN-III neurons were observed in the median preoptic nucleus and in the rostral perifornical area lateral to the PVN. The UCN-III fibers were distributed mainly in the hypothalamus and limbic structures. Hypothalamic regions that were innervated prominently by UCN-III fibers included the ventromedial nucleus, medial preoptic nucleus, and ventral premammillary nucleus. Outside the hypothalamus, the densest projections were found in the intermediate part of the lateral septum, posterior division of the bed nucleus stria terminalis, and the medial nucleus of the amygdala. Several major UCN-III terminal fields have been identified, including the lateral septum and the ventromedial hypothalamus, which are known to express high levels of CRH-R2. Thus, these anatomical data strongly support the notion that UCN-III is an endogenous ligand for CRH-R2 in these areas. These results also

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suggest that UCN-III is likely to mediate physiological functions, including food intake and neuroendocrine regulation (30). In the periphery, immunoreactive UCN-III is expressed in the human adrenals (31), heart, and kidney, particularly the distal tubules (32).

Animal studies using UCN-I-knockout mice suggest that UCN-I might not play a critical role in the HPA axis response to stress or in stress-induced autonomic control (33). However, when administered into the brain via the ventricles, UCN-I displays anorectic and anxiogenic properties similar to CRH. The UCNs are differentially distributed in the brain and periphery and appear to be involved in a rapidly expanding array of physiological mechanisms, particularly with respect to the appetite control and cardiovascular system, where they influence vascularization and angiogenesis (34–36).

An important component of the CRH/UCNs circuits appears to be a 37-kDa circulating protein capable of binding CRH and UCN-I, the CRH binding protein (CRH-BP) (37). In rodents, CRH-BP mRNA is expressed in the pituitary and the brain, especially the cerebral cortex, amygdala, bed nucleus of the stria terminalis, raphe nuclei, brainstem reticular formation, and olfactory, auditory, trigeminal, and vestibular sensory relay systems. Some of these sites also contain CRH and UCN-I-producing neurons or CRH/UCN target cells, such as the anterior pituitary corticotrophs. In humans, CRH-BP expression has been detected in brain, pituitary, liver, and placenta (38–40). The sites of overlapping expression of CRH-BP with CRH and UCN-I suggest that CRH-BP might modulate the synaptic or endocrine actions of CRH and/or UCN-I in the CNS and the pituitary. The biochemical properties of CRH-BP are well characterized; CRH-BP binds 40–90% of the total CRH, and CRH-BP levels are approximately 10-fold higher than CRH levels in most human brain regions (41). Despite this, the role of CRH-BP in health and disease remains unknown. What is known, however, is that CRH-BP blocks CRH-mediated ACTH secretion from anterior pituitary cultures (41, 42), suggesting that endogenous CRH-BP may act as a negative regulator of CRH *in vivo*, possibly playing a role in CRH clearance or degradation. This is supported by studies on transgenic mice, in which the response to constitutively elevated pituitary CRH-BP levels is a compensatory elevation of hypothalamic CRH and vasopressin, to maintain homeostasis in the HPA axis (43). In contrast, CRH-BP-deficient mice exhibit normal HPA axis function (44).

III. CRH-R Subfamilies

CRH and CRH-related peptides exert their actions in target cells via activation of two families of specific high-affinity CRH-R, termed R1 and R2, which are encoded by different genes (45, 46). Both of these genes are found in humans as well as in rodents and other mammals. In humans, CRH-R1 and -R2 are located on chromosomes 17 (17q12-q22) and 7 (7p21-p15), respectively, whereas in mouse, they are located on chromosomes 11 and 6, respectively (47–50). Interestingly, linkage disequilibrium (LD) and single nucleotide polymorphism studies have implicated the CRH-R1 gene in Parkinson's disease. This relates to a region of LD associated with late-onset Parkinson's disease on chromosome 17 that encompasses the CRH-R1 gene. Specific haplotype-tagging single nucleotide polymorphisms, lying in this region of LD, are located in or flank the CRH-R1 gene and are significantly associated with increased risk of neurodegeneration and Parkinson's disease (51). The CRH-Rs are membrane-bound proteins that belong to the family of seven transmembrane (7 TMD) G protein-coupled receptors (GPCRs) that, upon agonist binding, change their structural conformation and transduce signals across cells mainly through activation of heterotrimeric G proteins, which regulate a diverse network of intracellular systems.

The family of GPCRs is the largest single family of integral membrane proteins. Both CRH-R1 and CRH-R2 belong to the class B1 subfamily of GPCRs ("brain-gut" neuropeptide receptors). These receptors are encoded by 15 genes in humans, and the ligands for these receptors are polypeptide hormones of 27- to 141-amino acid residues. Structurally, CRH-R1 and CRH-R2 are approximately 70% identical at the amino acid level, but exhibit considerable divergence at the N terminus, consistent with their distinct pharmacological properties (see *Section IV.A*).

CRH-Rs are also found in nonmammalian vertebrate species. Studies on nonmammalian species (for review, see Ref. 52) have identified two receptor homologs closely related to the mammalian CRH-R1 and CRH-R2, from *Xenopus laevis* and chun salmon (*Oncorhynchus keta*). Studies of the puffer fish genome showed that *Fugu rubripes* also encodes two CRH-R homologs with significant similarity to human CRH-R1 and -R2, respectively. Unlike all other species studied, the *Ameiurus* catfish encodes two distinct mammalian CRH-R1 homologs and a single R2 ortholog. Thus, there has been extensive conservation in the expression of the two types of CRH-Rs in select organs during evolution. The two CRH-R1 homologs found in catfish showed greater similarity to each other than to the CRH-R2 homolog; therefore it is possible that the two catfish CRH-R1-like genes are likely derived from further gene duplication of the ancestral CRH-R1 gene. The CRH family of peptides exhibits significant sequence identity as well as primary- and secondary-structure similarity with the diuretic hormones in insects (53). These hormone peptides are essential for the regulation of fluid secretion by Malpighian tubules in insects. Their actions are also mediated via GPCRs homologous to the mammalian CRH-Rs. Functional studies suggest that CRH-related peptides coevolved with their GPCRs during the evolution of vertebrates and insects (53). Recent genomic analysis shows that both the mosquito (*Anopheles gambiae*) and fruit fly (*Drosophila melanogaster*) genomes encode orthologs of the diuretic hormone and diuretic hormone receptors. The two *D. melanogaster* diuretic hormone receptors showed 51 and 57% similarity to CRH-R1 and CRH-R2, respectively (54). These data provide us with a clear evolutionary trail for the origin of the CRH/CRH-R signaling system from invertebrates to vertebrates. Based on the concept that receptors and their agonists coevolved during evolution and that the evolution of most cell surface receptors in humans can be traced to invertebrates, it is likely that the ancestors of the diuretic hormone and diuretic hormone re-

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ceptors coevolved and gave rise to diuretic hormone/diuretic hormone receptor signaling in insects and CRH/CRH-R signaling in vertebrates. The latter might have originated as a paracrine signaling system important for osmoregulation. However, during early evolution of vertebrates, the ancestor CRH/CRH-R signaling pathway assumed additional functions that included the regulation of stress responses toward various environmental factors. The presence of multiple highly conserved CRH-like peptides and receptors in vertebrates suggests that gene duplication and the subsequent divergence of the regulatory mechanism of these paralogous genes provided an advantage during vertebrate evolution. Interestingly, CRH has been shown to regulate metamorphosis in response to pond drying in some amphibian species. This is consistent with the role of CRH family peptides as osmoregulators and as a stress transducer between the environment and the physiological responses of an organism. Therefore, the CRH family of peptides and their receptors are phylogenetically ancient developmental signaling molecules that allow developing organisms to coordinate physiological responses to a changing environment.

A. Distribution of CRH-R

CRH-R1 mRNA is widely expressed in mammalian brain and pituitary and is responsible for activation of the POMC gene and ACTH and β-endorphin release from the anterior pituitary. High levels of CRH-R1 are found in the cerebral cortex, cerebellum, amygdala, hippocampus, and olfactory bulb (55). In human peripheral tissues, CRH-R1 is expressed in a wide range of tissues such as the testis, ovary, endometrium, myometrium, placenta, adrenal, adipose tissue, skin, spleen, heart, and specific cells of the immune system (55–66). CRH-R2 mRNA is expressed in a discrete pattern in the brain, with highest densities in the lateral septal nucleus, bed nucleus of stria terminalis, VMH nucleus, olfactory bulb, and mesencephalic raphe nuclei (55). In addition, CRH-R2 is also widely expressed in peripheral tissues, with high levels in the skin and skeletal, smooth, and cardiac muscle (67, 68).

The distribution of CRH-R1 and CRH-R2 in the CNS and periphery is distinct and suggests diverse physiological functions, as implied by the phenotypes of the CRH-R1 or CRH-R2 knockout mice. Interestingly, mice deficient in CRH-R1 display decreased anxiety-like behavior and have an impaired stress response (69, 70), whereas CRH-R2 mutant mice have increased anxiety-like behavior, an accelerated HPA response to stress, and impaired cardiovascular function (71–73). Paradoxically, however, the responses to administration of CRH-R2 agonists and antagonists into specific brain regions reveal both anxiolytic and anxiogenic-like roles for CRH-R2 (for review, see Ref. 74). Also, it is important to note that the expression of CRH-R1 and -R2 in the periphery exhibits important species-related differences. Most studies in animal models suggest that the CRH-R2 is the main functional receptor expressed in peripheral organs (35, 36, 76, 77). In contrast, most human peripheral tissues express both CRH-R1 and -R2, indicating a higher level of complexity and more subtle roles for CRH and UCNs in human physiology and pathophysiology. From the available data, it would seem that the expression of both CRH-R1 and CRH-R2 in human peripheral tissues allows these peptides to exert diverse and sometimes contrasting effects. Certainly CRH and the UNCs play a role in physiological processes such as energy balance and metabolism, the maintenance of cardiac function and vascular tone, gastrointestinal motor function, reproduction, pregnancy and parturition, angiogenesis, and vascularization. It seems likely that they exert their actions via the activation of CRH-R1 or CRH-R2 or both (19, 21, 77–84), thus allowing CRH to fulfil its role as a stress-hormone integrating central and peripheral responses to stressful stimuli.

B. Splicing pattern and genomic organization

One of the more remarkable findings from the Human Genome Project (http://genome.wellcome.ac.uk) was the observation that human chromosomes harbor far fewer genes than were predicted. It appears that the key to the differences between humans and worms lies in the functions of some human genes and the proteins they encode. Furthermore, functional complexity is added by the use of alternative splicing of mRNA to create several distinct proteins from a single gene. Both CRH-R genes exhibit substantial similarity to the glucagon/PTH receptor (PTH-R) gene family that is characterized by the presence of introns within its TMD/cytoplasmic module, highly conserved cysteines in its extracellular domain (ECD), and a highly conserved first intracellular (IC) loop. The exon/intron junctions of the CRH, PTH, and glucagon receptor genes are remarkably similar in alignment following the signal peptide encoded by exons 1 and 2. The CRH-R1 exon/intron junctions are aligned to that of the PTH and glucagon receptors after exons 3, 5, 7, 8, 9, 10, and 12. CRH-R1 is similar to the PTH-R in that amino acids 457–509 are divided into exons 11 and 12, whereas this domain in the glucagon receptor is a single exon. The exon-intron organization of this GPCR family suggests a common evolutionary origin and, unlike other GPCR subfamilies, permits extensive alternate splicing. The evolutionary process has used this property to generate diverse receptors from a single gene, each with differing ligand specificity, binding, and G protein coupling. The fact that B1 GPCRs have multiple coding exons in comparison to other GPCR family members may be due to their being generated as an alternative to gene duplication during evolution. The increasing complexity seen within this GPCR subfamily seems to have evolved by divergence from a simpler regulated gene with increasing complexity of the organism.

1. CRH-R1. In most mammals, the fully active CRH-R1 receptor protein arises from transcription of all 13 exons present within the CRH-R1 gene sequence. In humans, however, the genomic organization and regulatory control is different, possibly reflecting a higher level of regulation associated with species evolution. In humans, the CRH-R1 gene, which spans over 20 kb, contains 14 exons, and the complete gene product is a 444-amino acid 7 TMD protein receptor, termed CRH-R1β, that exhibits impaired agonist-binding and signaling properties (45). Excision of exon 6, which encodes for a 29-amino acid insert in the first IC loop (IC1), from the mRNA transcript results in expression of

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CRH-R1α mRNA. This appears to be the main functional CRH-R1 receptor variant containing 415 amino acids, which primarily mediates CRH (and UCN-I) actions and is widely expressed throughout the body. Therefore, the CRH-R1β can be regarded as a "pro-CRH-R1" receptor isoform, with unknown, if any, physiological function. Although lack of CRH-R1β receptor isoform-specific antibodies has prevented the conclusive demonstration of CRH-R1β protein expression in native cells, mRNA studies suggest that this CRH-R1 variant is expressed in the pituitary (45) as well as in peripheral tissues such as the myometrium (59), mast cells (85), endometrium (86), and heart (our unpublished data). Thus far, no tissue has been identified expressing CRH-R1β alone, suggesting that the splicing mechanism is closely related to the mechanism regulating CRH-R1 gene expression in native tissues. One interesting feature of CRH-R1β is that it is only expressed in humans. In rodents, the exon 6 encoding the IC loop characteristic of CRH-R1β is absent, and, as mentioned above, the mouse and rat CRH-R1 gene contains only 13 exons (87). This suggests that the CRH-R1β variant does not perform a crucial physiological role. This type of splicing mechanism, utilizing specific exon/intron splicing sites and excision of exons to generate multiple receptor variants, is not unique to CRH-R1, but is rather shared by other members of the B1 receptor superfamily. For example, a similar calcitonin receptor (CT-R) variant has been identified in a giant cell tumor of bone that contains a similar 16-amino acid insert in IC1 and has reduced signaling properties compared with the fully active receptor variant (88). The insert sequences in IC1 of both CRH-R1β and CT-R "long" variant contain highly charged amino acids in their insert C termini proximal to the IC1/TMD2 junction, which might be responsible for the impaired ability to transmit signals across the cells. Just why these mRNA variants have been conserved through evolution is unknown.

The CRH-R1 gene appears to be subject to significant alternative splicing, and a growing number of CRH-R1 mRNA splice variants have been described in humans and other species. Although, as mentioned above, their physiological role is questionable, structural analysis has provided us with some useful models for studying the structural determinants of the CRH-R1 functional characteristics in comparison with other members of the B1 family of GPCRs. These variants are generated by various partial or complete exon deletions, some of which are associated with a frame-shift in the open reading frame (63, 89, 90, 92). These variants have been termed R1c-n, and all have exon 6 spliced out together with other deletions. CRH-R1c is missing exon 3, and therefore 40 amino acids are missing from the N terminus, including two regions critical for high-affinity ligand binding (see *Section IV.B*). Mutations in this region can affect CRH binding (93); thus, it is not surprising that expressed recombinant CRH-R1c has a decreased CRH binding capacity. CRH-R1d, which has been identified in humans and hamsters, has exon 13 (exon 12 in the rodent R1 homolog) deleted, which leads to the loss of 14 amino acids from the C-terminal end of the putative 7 TMD. This might result in either a short 7 TMD where the proximal residues of the C-tail are drawn into the membrane or a 6-TMD receptor variant containing a protein segment that fails to segregate into the membrane lipid bilayer leading to an EC C terminus, similar to the CT-R variant Δe13 (94). Overexpression studies of recombinant CRH-R1d have demonstrated that, although this CRH-R1 variant retains agonist binding characteristics, it has significantly impaired G protein coupling and signaling properties. Given that most sites involved in GPCR signaling through posttranslational modifications and docking of signaling proteins are in the carboxyl-terminal tail, the possible retraction and distortion of this tail induced by the 14-amino acid sequence deletion would be expected to alter signaling and hence function. Similar splice variants arising from the same exon deletions have been described for other members of the B1 subfamily of GPCRs, such as the PTH-R (95) and the type II receptor for vasoactive intestinal peptide and pituitary adenylate cyclase activating polypeptide receptor for vasoactive intestinal peptide (96). Analysis of the nucleotide sequence reveals that there are conserved splicing sites within the 7 TMD (site of exon deletion), shared among the members of this receptor family. The structural features of the receptor splice variants found in humans are shown in Fig. 1.

The work of Pisarchik and Slominski (63, 92) in human, mouse, and hamster has expanded the list of potential CRH-R1 mRNA splice variants involving the excision of multiple exons, which has profound consequences for the tertiary structure of any translated protein. Currently, eight additional mRNA transcripts have been identified, named R1e-n, each with a unique exon splicing pattern and predicted protein structure. The functional significance of these novel transcripts is uncertain.

The physiological relevance of the CRH-R1 mRNA splice variants has been dismissed as being a result of aberrant transcription. Characterization of the biological activity and signaling properties of some of these CRH-R1 variant receptor proteins is based on heterologous overexpression systems using recombinant protein expression and not native cells. This is due to a lack of suitable methods to demonstrate endogenous CRH-R1 variant expression and to study their physiological roles, if any, in mammalian tissues. Nevertheless, the complex pattern of CRH-R1 alternative splicing that is tissue-specific and physiological process-specific [*i.e.*, expression of myometrial CRH-R1d at term of human pregnancy (90)] and affected by environmental stimuli [*i.e.*, expression of CRH-R1g after UV irradiation (63)] suggests a functional role in modifying CRH actions in target tissues. It is conceivable that the presence of different isoforms could have a substantial influence on cellular response to CRH and CRH-like agonists. Regardless of whether or not these aberrant transcripts are significantly expressed as protein products, the dominant expression of the aberrant transcripts at the expense of the wild-type receptor at transcription would have the end result of reducing levels of functional receptor. Although our current knowledge of the mechanisms regulating CRH-R1 splicing and the specific function of distinct CRH-R1 variants is poor, emerging evidence points toward potentially important biological mechanisms. For example, we have identified a specific mechanism involving steroid hormones, which regulates the ratio of CRH-R1α/R1β mRNA expression in human myometrial smooth muscle cells during pregnancy. This may represent a mechanism for regulating tissue responsiveness to CRH (Fig. 2) (97). In this

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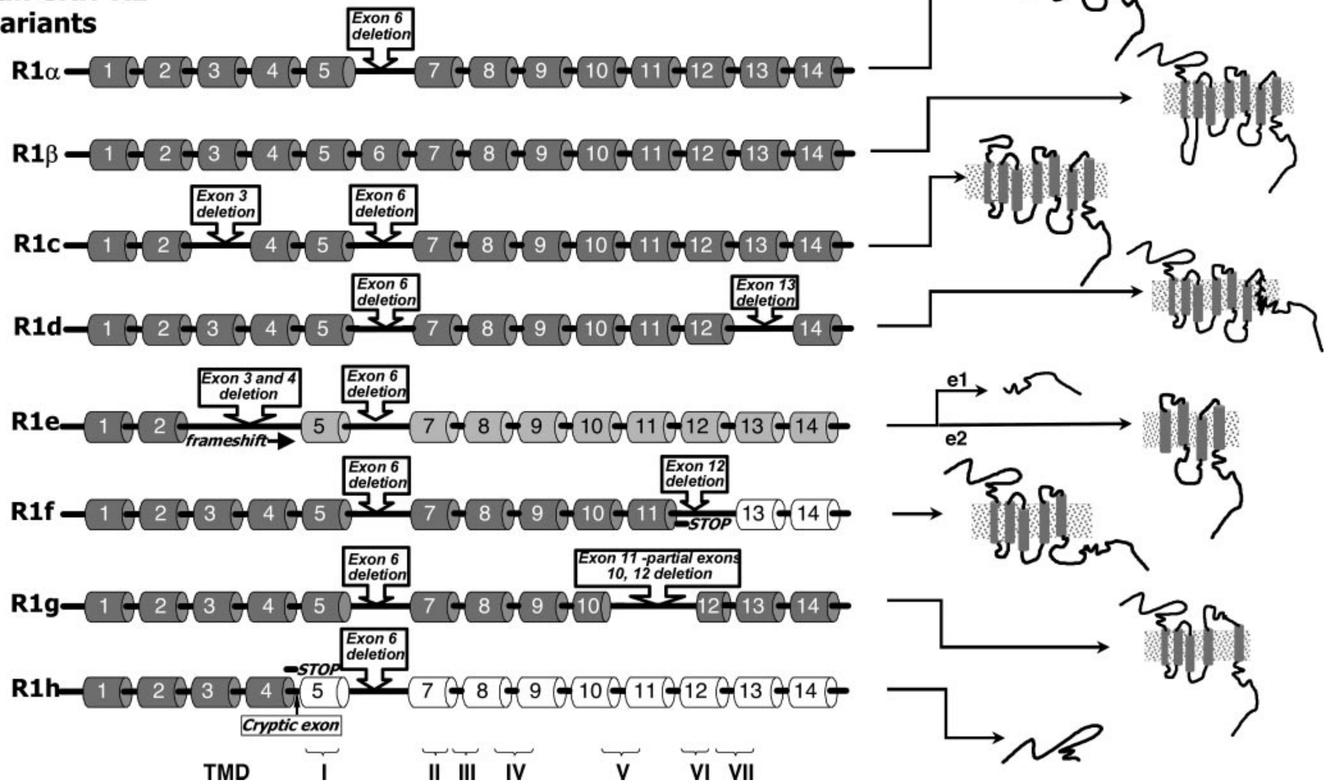

FIG. 1. Schematic representation of exon structure of human CRH-R1 variants and structural differences of potential protein products.

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example, progesterone alters the transcription of the CRH-R1 gene in such a way that the predominant transcript encodes for CRH-R1α, thus enhancing tissue responsiveness to CRH, an effect that is blocked by estrogen. Furthermore, some variants of CRH-R1, such as the CRH-R1d, fully retain the ability to bind agonist with high affinity although they are deficient in signaling. These receptor variants might act as "decoy receptors" capable of competing with the full-length receptors for agonist binding and absorbing CRH and CRH-like peptide bioactivity and therefore, change the efficiency of hormonal stimulation. Interestingly, in other receptor systems, such as the CT-R system, similar splice variants containing deletions in the 7 TMD, when coexpressed with the fully active CT-R variant, act as dominant negative regulators by forming heterooligomers within the cell. This process inhibits normal receptor expression at the cell surface, leading to a reduction in the signaling response (98). Similar interactions have been observed with the truncated forms of CRH-R1, e and h, which are capable of binding CRH and influencing tissue responsiveness to CRH. For example, in coexpression experiments, CRH-R1e attenuated and CRH-R1h amplified CRH-R1α signaling (99). These observations provide tantalizing, although far from conclusive, evidence suggesting that the regulation of transcription and splicing of the CRH-R1 receptor gene might play a major role in determining tissue responsiveness to CRH and UCNs. It should be remembered, however, that there is no evidence that these transcripts are translated into functional protein. This might not be a prerequisite, however, because the dominant expression of the aberrant transcripts at the expense of the wild-type receptor at transcription would have the end result of reducing levels of functional receptor.

With these caveats in mind, Pisarchik and Slominski (92) have proposed a division of all potential CRH-R1 variants into four groups according to their potential impact on agonist signaling (Fig. 3):

a. Group 1. The first group includes variants with no frameshift (β, c, d, g, and n) but with a variable number of TMDs and intra- or extracellular C terminus (yet to be conclusively demonstrated for some variants) that differ in their agonist binding and G protein-coupling characteristics.

b. Group 2. The second group consists of mRNA variants missing exons 1, 2, 3, and 4, which are important for agonist binding and therefore appear functionally inert (R1e). Although it is difficult to attribute any direct role to individual members of this group, they might have important functional significance by modulating CRH-R1α activity.

c. Group 3. Members of the third group have conserved the original reading frame of CRH-R1 up to the fifth exon, thus retaining an intact CRH-binding domain but having no TMDs. Two members of this group have been identified: CRH-R1j and CRH-R1h. CRH-R1j has exon 5 deleted,

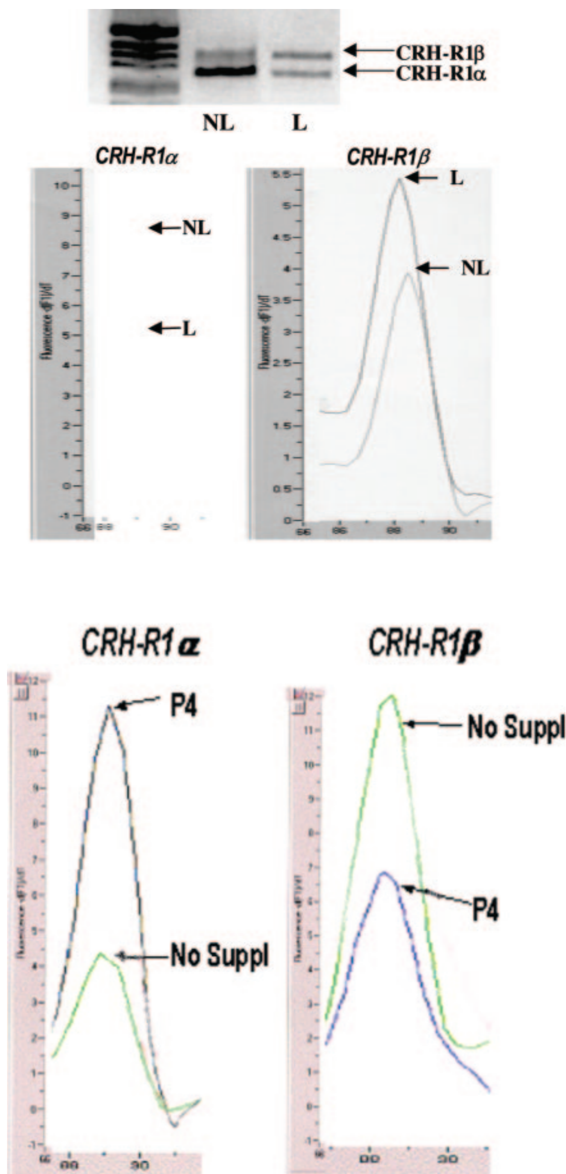

FIG. 2. Examples of differential splicing mechanisms regulating CRH-R variant expression. In this set of experiments, quantitative RT-PCR with specific oligonucleotide primers was used. *Top*, CRH-R1α and -R1β mRNA expressed in human myometrial tissue obtained at term before the onset (NL) and during labor (L). Results are presented as maxima of melting curves of CRH-R1α (89.20 C), and CRH-R1β (88.56 C) genes. Similar results were obtained from six independent myometrial biopsies. *Bottom*, CRH-R1α and -R1β mRNA expressed in human myometrial cells treated with or without progesterone (P_4; 5 μM) for 16 h. These results represent four independent myometrial cell preparations.

whereas CRH-R1h has an insertion of a cryptic exon between exons 4 and 5. If translated, these isoforms could potentially serve as soluble CRH-BPs or modulate agonist binding.

d. Group 4. The fourth group consists of mRNA variants that have an intact CRH-binding domain and a variable number of TMDs (CRH-R1f, k, and m). Each of these transcripts contains a frame-shift that potentially alters their C terminus, raising questions about their potential for participation in signal transduction.

Studies on the promoter of the human CRH-R1 gene suggest that CRH-R1 agonists such as CRH and UCN-I can positively regulate CRH-R1 gene transcription (100) in agreement with several animal studies (101–104). The hypothalamic PVN is a site of CRH-induced up-regulation of CRH-Rs. An example is the autoregulation of CRH biosynthesis in the PVN through up-regulation of CRH-R1, showing the mediation of positive effects on the amygdaloid CRH system (101). In rats, CRH-R1 mRNA in the PVN is increased after stress, and this response is attenuated by central CRH blockade. Elevated levels of central CRH may trigger CRH-R1 mRNA transcription in the PVN, hippocampus, and frontal cortex (102), suggesting a positive feedback of CRH on its own receptor, acting as a functional adaptation of the HPA axis in response to stress (103). These animal data are supported by studies on human cells and the human CRH-R1 promoter (100) in which a transcriptional positive feedback effect was shown to be dependent on activation of both protein kinase A (PKA) and protein kinase C (PKC) in the case of CRH, and PKC alone in the case of UCN. Collectively, the data suggest that the CRH-R1 gene is under the influence of both CRF and UCN, acting via distinct signaling pathways to create a positive feedback loop and regulate further the transcription of the receptor. However, this feedback mechanism requires further characterization because there is conflicting evidence suggesting positive and negative effects of CRH on rat anterior pituitary CRH-R1 expression (104, 105). Glucocorticoids are another important regulator of CRH-R1 gene expression because dexamethasone has been shown to act as a negative regulator of CRH-R1 mRNA expression in rodent anterior pituitary and PVN (106–109).

Interestingly, the human as well as the rat CRH-R1 promoters are TATA-less, suggesting that Sp1 sites might play a crucial role for driving transcription because there are a high number of potential Sp1 elements clustered around the transcriptional start sites (100). Other members of the B1 family of GPCRs share this feature, including the promoters for the CT-R, the glucagon-like peptide receptor-1 and the vasointestinal peptide 1 receptor genes (100). The promoter region of the CRH-R1 also contains sites for Egr 2 (Krox-20) and YY1 (yin-yang 1) transcription factors (members of the zinc finger DNA binding transcription factors), which may also play a role in controlling transcription. The presence of a putative transcription response element, however, should not be taken as proof that potential binding factor is a regulatory factor.

2. CRH-R2. Interestingly, the CRH-R2 gene exhibits a completely different splicing pattern compared with CRH-R1, possibly relevant to its distinct role in mammalian physiology. Mammals express three known CRH-R2 variants: CRH-R2α and -R2β are found in both human and rodents, and -R2γ has so far been found only in the limbic regions of the human CNS (110–112), although genomic clones containing the CRH-R2γ subtype have been identified in the olive baboon and chimpanzee. All three variant mRNAs are produced by the use of an alternate 5′ exon 1 that splices onto a common set of downstream exons (2–12), resulting in R2 variants, with identical transmembrane and C-terminus domains. These variants differ only in their N-terminal ECDs; CRH-R2α has

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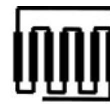

CRH-R1 variants

Group	characteristics	variants	Structural differences
1st	various insertions/ deletions with variable number of TMD and intra-/extra-cellular C-tail no frameshifts	β c d g n	444aa (extra 29aa in the IC1) 375aa (40aa missing from N-terminus) 401aa (14aa missing from TMD7) 341aa (missing TMD 5, 6 and IC3) 327aa (missing TMD5,6,7 , IC3 and EC3)
2nd	missing agonist binding domain-functionally inert?	e	91aa (first 40aa from N-terminus/unique seq)-e1 240aa (4TMD/C-tail)-e2
3rd	intact agonist-binding domain, no TMDs (*potential soluble proteins*)	j h	172aa (N-terminus) 145aa (N-terminus)
4th	intact agonist-binding domain, variable number of TMDs	f k m	370aa (N-terminus/1-5TMD/unique C-tail) 339aa (N-terminus/1-4TMD/unique C-tail) 356aa (N-terminus/1-4TMD/unique C-tail)

FIG. 3. Proposed classification of potential CRH-R1 variants according to their potential impact on agonist signaling. aa, Amino acids. [Derived from Ref. 92.]

34 amino acids at the N terminus, which are replaced by 61 amino acids to form the CRH-R2β or 20 amino acids to form the CRH-R2γ. The human CRH-R2 gene spans 50 kb and consists of 15 exons. Exon β1a contains the 5′-untranslated region and the start codon for CRH-R2β and, together with exon β1b, contributes to the N-terminal ECD. Exons γ1 and α1 contain the 5′-untranslated region and start codon for CRH-R2γ and CRH-R2α, respectively, which contributes to their unique N-terminal ECD. The remainder of the N terminus is encoded on exons 2–7, with exons 7–11 coding for the 7 TMDs and exon 12 coding for the intracellular C terminus, all of which are common to all known functional CRH-R2 receptors (113). The different N termini do not significantly alter agonist binding and signaling properties of the various CRH-related peptides, although the CRH-R2β is about 10-fold more potent in second messenger activation compared with CRH-R2α or R2γ (112). These variants, however, do exhibit significant differences in their tissue distribution. The CRH-R2α and the CRH-R2β are expressed in both the brain and periphery (114), although CRH-R2α is mainly localized to the subcortical structures (114), whereas the CRH-R2γ appears to be confined predominantly to the brain (112). In addition, both the CRH-R2α and CRH-R2β variants are differentially expressed in heart, skeletal muscle, and myometrium (59, 77). In rodents, the CRH-R2α is the predominant CRH-R2 variant expressed in the rat brain (112, 114), whereas CRH-R2β mRNA is widely expressed in peripheral tissues, with highest levels in the skeletal muscle, heart, and skin (67). Interestingly, the CRH-R2β variant is primarily expressed peripherally in rodents, whereas the CRH-R2α is the predominant splice variant found in the periphery of humans. Given that different promoters regulate each CRH-R2 variant expression (see below), this diversity could be related to the regulation of expression at different tissues. The CRH-R2 gene appears to have diverged from CRH-R1 by duplication and then by increasing complexity to produce three subtypes with different pharmacology and differential expression. CRH-R2α is present in amphibians, whereas in rodents a second variant CRH-R2β is also present, and in primates a third variant CRH-R2γ.

The arrangement of the CRH-R2 gene provides the potential for generating multiple alternate splice forms of mRNA (111–113). It is predicted that the CRH-R2β pre-mRNA is differentially spliced, producing multiple alternate splice forms, and if any permutation of exons between β1a and 2 occurs, at least 12 alternate splice forms can be produced, some with high levels of expression (113). Different types of aberrant mRNA splice variants have also been reported: 1) a truncated CRH-R2α mRNA isolated from rat amygdala (CRH-R2α-tr), which encodes for the first three TMDs and a part of the fourth TMD of the CRH-R2α and binds CRH, but not UCN-I, with almost the same affinity as CRH-R2α (115); and 2) a soluble CRH-R2α, in which exon 6 is deleted and translation of this variant produces a predicted 143-amino acid soluble protein. The translated protein includes the majority of the first ECD of the CRH-R2α followed by a unique 38-amino acid hydrophilic C terminus resulting from a frame-shift produced by deletion of exon 6. The soluble CRH-R2α variant has high levels of expression in the olfactory bulb, cerebral cortex, and midbrain regions. Experiments using recombinant proteins showed that soluble CRH-R2α protein inhibits cellular responses to CRH and UCN, supporting a potential role as a biological modulator of CRH and CRH-related peptides (116).

Three distinct promoters and differential splicing (113) control regulation of the human CRH-R2 gene expression.

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The arrangement of the 5′-flanking region of the gene is similar to that of the CRH-R1 gene in that it lacks a functional TATA or CCAAT box and contains several specificity protein 1 binding elements, which suggests that these elements constitute the minimal promoter. Each of the promoters contains some consensus regulatory sequences that provide some insights into CRH-R2 physiology. For example, the CRH-R2β and CRH-R2α promoters contain consensus sequences for binding myogenic transcription factors, such as the myocyte-specific enhancer MEF-2, which may be involved in the regulation of expression in cardiac, skeletal, uterine, and smooth muscle. In contrast, the CRH-R2γ promoter contains a putative regulatory element for the pituitary-specific factor Pit-1a, raising the possibility that the CRH-R2γ is the CRH-R2 variant expressed in the pituitary (117). Putative glucocorticoid-responsive elements are also present in the CRH-R2α promoter, consistent with studies showing glucocorticoid regulation of CRH-R2 expression in the heart and brain (118, 119). This mechanism appears to be conserved across species because the mouse CRH-R2α 5′ flanking region contains 23 putative half-palindrome glucocorticoid response element sequences within its 2.4-kb sequence (120). This might explain the finding that hypothalamic murine CRH-R2α gene transcription is inhibited by glucocorticoid administration *in vivo* and enhanced by adrenalectomy. However, other studies have reported that in the VMH nucleus, the levels of CRH-R2 mRNA are up-regulated by corticosterone (119) and leptin administration (121), whereas, stressful stimuli such as starvation (119), repeated immobilization stress (122), and maternal deprivation (123) decrease the expression level of CRH-R2 mRNA in the rat VMH. The same study suggested that CRH-R2 gene expression is differentially regulated in different hypothalamic nuclei because neither corticosterone administration, starvation, nor adrenalectomy influenced the levels of CRH-R2 mRNA in the hypothalamic PVN (119). Clearly, additional studies are required to clarify these discrepancies and reveal the manner by which the CRH-R2 pathway is involved in physiological responses to stress in normal and transgenic mice models.

Despite the well-established role of CRH-Rs in human physiology and nosological states such as clinical depression, no striking abnormalities in CRH-R expression and/or function in human disease have been observed, with a few notable exceptions. In ACTH-secreting pituitary adenomas, a significant overexpression of the CRH-R1 mRNA has been reported (124), which may contribute to a disturbed receptor regulation. Also, in brains from suicide victims, the mRNA for cerebral cortical CRH-R1, but not CRH-R2, appears to be reduced, possibly secondary to sustained increase of CRH activity (125, 126), supporting the view that only the CRH-R1 subtype is aligned with mood disorders. The human placenta in patients with preeclampsia is another example of an organ where abnormal expression of CRH-R1 and -R2 has been reported and linked to an increased placental CRH production and abnormal regulation of the feto-placental blood flow (127, 128). However, in these cases it remains to be established whether abnormalities in CRH-R expression contribute to the pathogenesis of the disease or are secondary to the illness or the stressors associated with it.

IV. Agonist-CRH-R Interaction

A. Receptor pharmacology

Despite an overall similarity of greater than 70% at the amino acid level, the two types of CRH-R exhibit different pharmacological characteristics, reflecting their unique and sometimes complementary or contrasting roles in specific tissues. The low homology at the extracellular N terminus (approximately 47%) accounts for the distinct ligand-specificity characteristics because the N terminus is primarily responsible for agonist binding (see *Section IV.B*). CRH-R1 binds CRH as well as UCN-I, but not UCN-II and -III, with equivalent high affinity. However, the CRH-R2 exhibits ligand selectivity and binds all the UCNs with significantly higher binding affinity than CRH, suggesting that these peptides may be its natural ligands. The rank order of mammalian CRH-like peptides for binding affinity at CRH-R1 receptors is: UCN-I > h/rCRH > α-helical CRH (9–41) (a peptide antagonist); however, at the CRH-R2α receptors, the binding affinity rank order is: human (h) UCN-I = hUCN-II > astressin = astressin$_2$-B = antisauvagine-30 > hUCN-III > α-helical CRH (9–41) > h/rCRH $\gg$ ovine (o) CRH. oCRH differs from its human homolog by eight amino acid residues and, in cellular systems expressing both types of CRH-R, it is the agonist of choice for the selective stimulation of CRH-R1, because it preferentially binds to CRH-R1. The binding constants of oCRH for CRH-R1 and -R2 differ by two orders of magnitude (129, 130). Recently, a CRH-R1-specific peptide agonist was generated, named cortagine ([Glu^{21},Ala^{40}] [Svg$^{1-12}$] × [h/rCRH$^{14-30}$] × [Svg$^{30-40}$]), by synthesis of chimeric peptides derived from h/rCRH, oCRH, and sauvagine (Svg; a frog CRH-related agonist) (131), and this might allow better characterization of CRH-R1 and -R2 function.

Characterization of the structure-function relationship of CRH and CRH-related agonists has revealed the presence of three main functional domains (132–134) (Fig. 4). The first domain (residues 1–16) is responsible for both binding and receptor activation. The second domain (residues 17–31) appears to function as a linker providing the appropriate spatial and conformational support for the two binding regions located in domains 1 and 3 and contains the CRH-BP binding site (stretch of amino acid residues 22–25, Ala-Arg-Ala-Glu, of h/rCRH representing the ARAE motif) (135). Finally, the third domain, consisting of residues 32–41, is important for receptor binding. Sequence analysis studies of all the members of the family of CRH-related agonists have revealed a number of differences between the CRH-R2 selective agonists, UCN-II and UCN-III, and the CRH-R nonselective agonists, CRH and UCN-I. A residue in the first domain, proline at position 11 (the numbering of residues is based on h/rCRH sequence), is found only in CRH-R2 selective peptides and might play an important role in determining receptor selectivity because substitution of Pro^{11} in the hUCN-II sequence with corresponding amino acids, found in the CRH-R nonselective agonists, decreased binding potency to CRH-R2 while increasing CRH-R1 activity. It is likely that the presence of this proline residue in the first domain alters the α-helix structure of the peptide, because proline is the stron-

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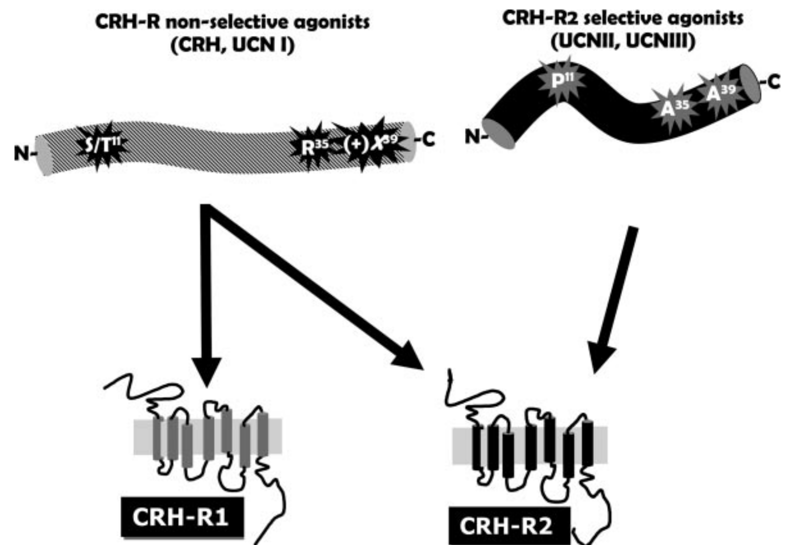

FIG. 4. Schematic representation of amino acids within the CRH/CRH-related agonists sequence important for determining CRH-R subtype selectivity. It is now accepted that amino acid residues 32–41 are important for receptor binding, whereas residues 1–16 are responsible for both binding and receptor activation. Residues present in the domain 17–31 appear to function as a linker providing the appropriate spatial and conformational support for the two binding regions. CRH-R2 selective agonists contain a proline at position 11 and alanine residues at positions 35 and 39 (the numbering of residues is based on h/rCRH sequence). In contrast, CRH-R nonselective peptides contain an arginine at position 35 and an acidic amino acid at position 39.

gest modifier of α-helix structure and a promoter of turn motifs. Consequently, the presence of Pro^{11} in the sequence of UCN-II and UCN-III may indicate that the α-helix present in the first domain of the nonselective CRH-R peptides is modified through the introduction of a kink or turn motif, a modification that may be important in determining agonist selectivity (136). Indeed, circular dichroism spectroscopy studies support the view that the presence of Pro^{11} in this region decreases α-helicity and impairs binding to CRH-R1 (137). Furthermore, in the third domain of the CRH-related peptides, CRH-R2 selective peptides contain alanine residues at positions 35 and 39, whereas CRH-R nonselective peptides contain an invariant arginine at position 35 and an acidic amino acid at position 39. Introducing proline at position 11 and alanine at positions 35 and 39 results in increased CRH-R2 selectivity in CRH-R nonselective peptides, mainly through the loss of CRH-R1 potency. Interestingly, unlike sauvagine and hUCN-I, this substitution in the h/rCRH sequence results in a loss of potency at both the CRH-R1 and -R2 (136), indicating that CRH requires additional substitutions to achieve substantial CRH-R2 selectivity.

B. Receptor-agonist interaction: the role of the N- and J-domains

Like all class B1 GPCRs, the CRH-Rs possess a large ECD that allows recognition and high-affinity binding to the carboxyl-terminal regions of peptide ligands. This interaction alone is not sufficient to stimulate coupling of the receptor to G proteins, and an additional interaction is required between the juxta-membrane domain of the GPCR (the transmembrane helices and intervening loops J-domain) and the first few residues within the amino-terminal portion of the peptide ligand to induce intracellular signal activation. Based on this, the generation of N-terminus-truncated CRH peptides produces high-affinity competitive antagonists for CRH-R [*e.g.*, α-helical CRH (9–41)]. Additional modifications have produced a number of different antagonist peptides including astressin [cyclo(30–33)-[D-Phe^{12}, $Nle^{21,38}$, Glu^{30}, Lys^{33}]CRF-(12–41)], a high-affinity antagonist for both CRH-R receptors with enhanced biological stability (138) and no detectable agonist activity for the CRH-R1.

Mass spectrometric analysis of a soluble form of the N terminus of the human CRH-R1 yielded a 1:1 complex with ligand, and analysis of the disulfide bond arrangement revealed bonds between Cys^{30} and Cys^{54}, Cys^{44} and Cys^{87}, and Cys^{68} and Cys^{102} (139) (Fig. 5A). An identical arrangement was also found in the soluble N terminus of the mouse CRH-R2β (140). This arrangement is similar to that of the N terminus of the PTH-R, suggesting a conserved structural motif in the N-terminal domain of the B1 family of GPCRs (141). Recent studies using nuclear magnetic resonance have provided valuable insights into the three-dimensional (3D) structure of the N-terminal ECD of the mouse CRH-R2β (142). This technique has identified a short consensus repeat (SCR) in this domain, which is more commonly found in proteins of the complement system. This model proposes that the N terminus captures the C-terminal segment of the ligand, allowing the N terminus to penetrate into the TMD region of the receptor to initiate signaling. Key residues of the SCR in the ECD1 are conserved in the GPCR subfamily, suggesting the importance of this SCR fold in the ECD1s of this subfamily.

According to studies by Grace *et al.* (142), the CRH-R2β N terminus contains two antiparallel β-sheets comprising residues 63–64 (β1 strand), 70–71 (β2 strand), 79–82 (β3 strand), and 99–102 (β4 strand). This polypeptide fold is stabilized by three disulfide bonds between residues Cys^{45}-Cys^{70}, Cys^{60}-Cys^{103}, and Cys^{84}-Cys^{118} and by a central core consisting of a salt bridge involving Asp^{65}-Arg^{101}, sandwiched between the aromatic rings of Trp^{71} and Trp^{109}. The two β-sheets, interconnected by this core, form the scaffold flanked by two disordered regions (residues 39–58 and 84–98) (Fig. 5B). Furthermore, the core is surrounded by a second layer of highly conserved residues, Thr^{69}, Val^{80}, and Arg^{82}, and conservatively conserved residues, Thr^{63}, Ser^{74}, and Ile^{67}. Other conserved residues, such as Pro^{72} and Pro^{83}, might be important for ending the β-strands, as well as Gly^{77}, Asn^{106}, and Gly^{107} located in the hinge regions of the two β-sheets, which may be important for their relative orientation. An-

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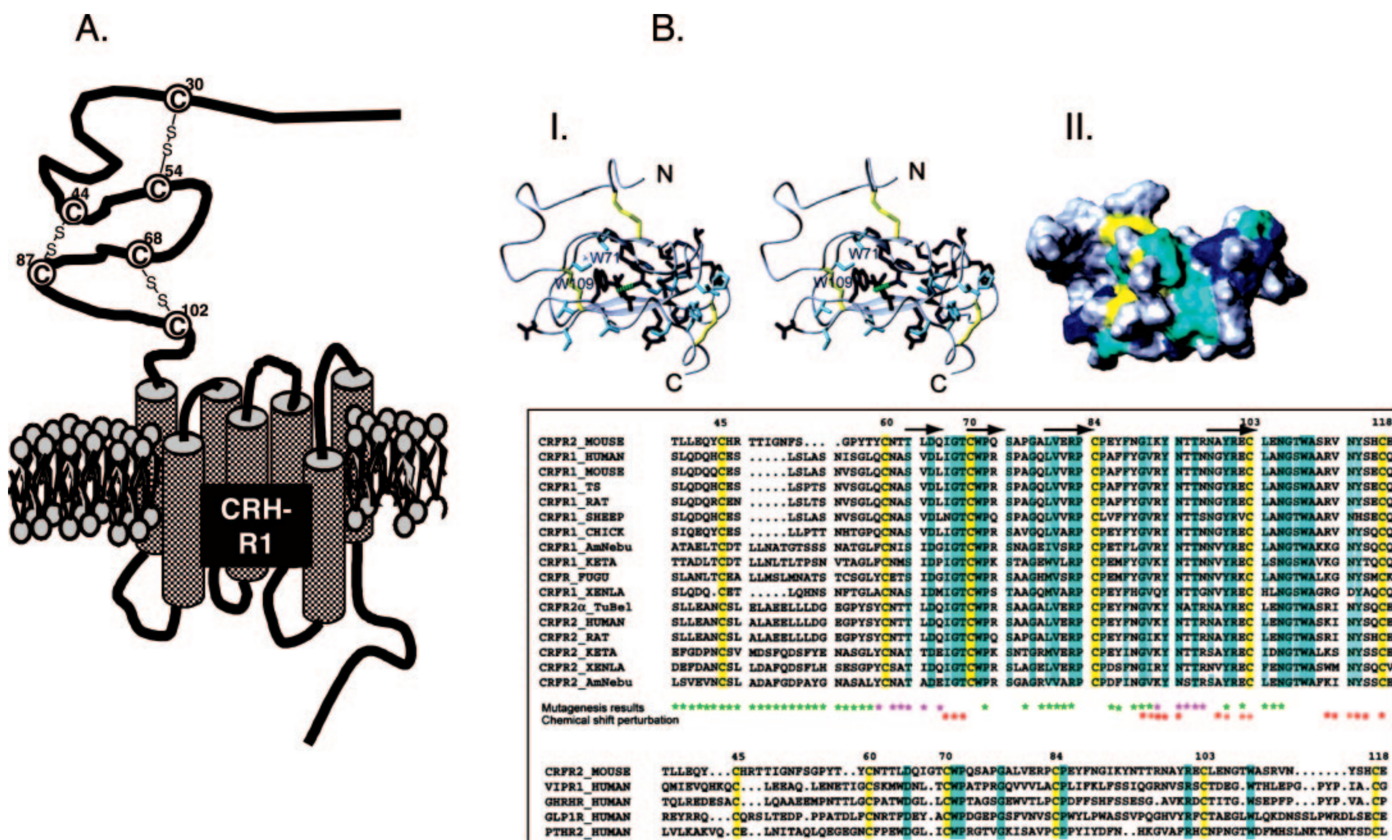

FIG. 5. A, Schematic of the disulfide bond arrangement between Cys^{30}-Cys^{54}, Cys^{44}-Cys^{87}, and Cys^{68}-Cys^{102} in the N terminus of the CRH-R1 receptor, based on mass spectrometric analysis of a soluble form of the N terminus of the human CRH-R1 (139). B, Mapping the conserved amino acids, as shown in Ref. 142, onto the 3D structure of ECD$_1$-CRH-R2β. Stereo view (I) and surface view (II) of the 3D structure with side chains of the conserved amino acids within the B1 family of GPCRs colored *dark blue* and similar residues colored *light blue*. The salt bridge between Asp^{65} and Arg^{101} is shown by the *green dashed line*. *Inset*, Sequence alignment of the ECD$_1$ of the CRH-R family and B1 GPCR family. Only a representative set of sequences is shown. Conserved cysteines are highlighted in *yellow*, conserved amino acids throughout the whole B1 family are *blue*, and amino acids conserved more than 80% throughout the whole B1 family are *light blue*. Mutagenesis studies for the identification of receptor-ligand interaction are summarized here: *magenta stars* represent amino acid segments proposed to be involved in hormone binding, and *green stars* represent amino acid segments that are less important for binding. The β-sheet secondary structure elements are labeled by an *arrow* above the sequence. TS, Treeshrew; AmNebu, *Ameriurus nebulosus*; TuBel, *Tupaia belangeri*; VIPRI, vasoactive intestinal peptide receptor 1; GLPIR, glucagon-like peptide 1 receptor; PTHR2, PTH receptor 2. [Reproduced with permission from C. R. Grace *et al*.: *Proc Natl Acad Sci USA* 101:12836–12841, 2004 (142). © National Academy of Sciences USA.]

other cluster of conserved residues is present in the disordered loop between strands β3 and β4 (Gly^{92}, Phe^{93}, Asn^{94}, and Thr^{96}). In contrast, the disordered loop from residues 39–58 is highly variable in amino acid sequence.

Astressin B has been used in nuclear magnetic resonance chemical-shift perturbation experiments to identify the CRH-R2β ligand binding site (142). The largest chemical-shift perturbations were observed in the segments comprising residues 67–69, 90–93, 102–103, and 112–116. These residues are clustered in the cleft region between the tip of the first β-sheet and the edge of the "palm" of the second β-sheet. The observed changes in the chemical shifts in the disordered loop region 85–98 suggest that folding occurs after ligand binding. Circular dichroism spectroscopy data support this view and show a conformational change toward a more structured N terminus upon ligand binding (140). Furthermore, the accumulated distribution of positive charges on the "back side" of the structure (Arg^{47}, Arg^{82}, Arg^{97}) is indicative of orientation toward the negatively charged ECDs 2–4 and the transmembrane segment of CRH-R2β. Based on these observations, it has been proposed that hormone binding and receptor activation occur in two steps. First, the ligand binds with its C-terminal segment to the solvent exposed binding site of the N terminus. In order for an agonist to elicit a signaling response, its N-terminal segment penetrates into the transmembrane segments of the CRH-R, producing activation of the receptor. In contrast, antagonists, like astressin, lacking the first 11 N-terminal residues critical for receptor activation cannot penetrate the transmembrane region and activate the receptor (Fig. 6).

The J-domain also appears to participate in agonist/antagonist interactions with CRH-R2. Studies using the subtype 2α of the rat CRH-R2 (143) revealed that the juxtamembrane receptor domain determines the selectivity of antisauvagine-30 [a synthetic CRH-R2 antagonist (144)], whereas the N-terminal-ECD contributes to selectivity of UCN-III, and both domains contribute to selectivity of UCN-II and astressin$_2$-B [CRH-R2 antagonist (145)]. Therefore, ligands differ in the contribution of receptor domains to their selectivity, and CRH-R2-selective antagonists can bind

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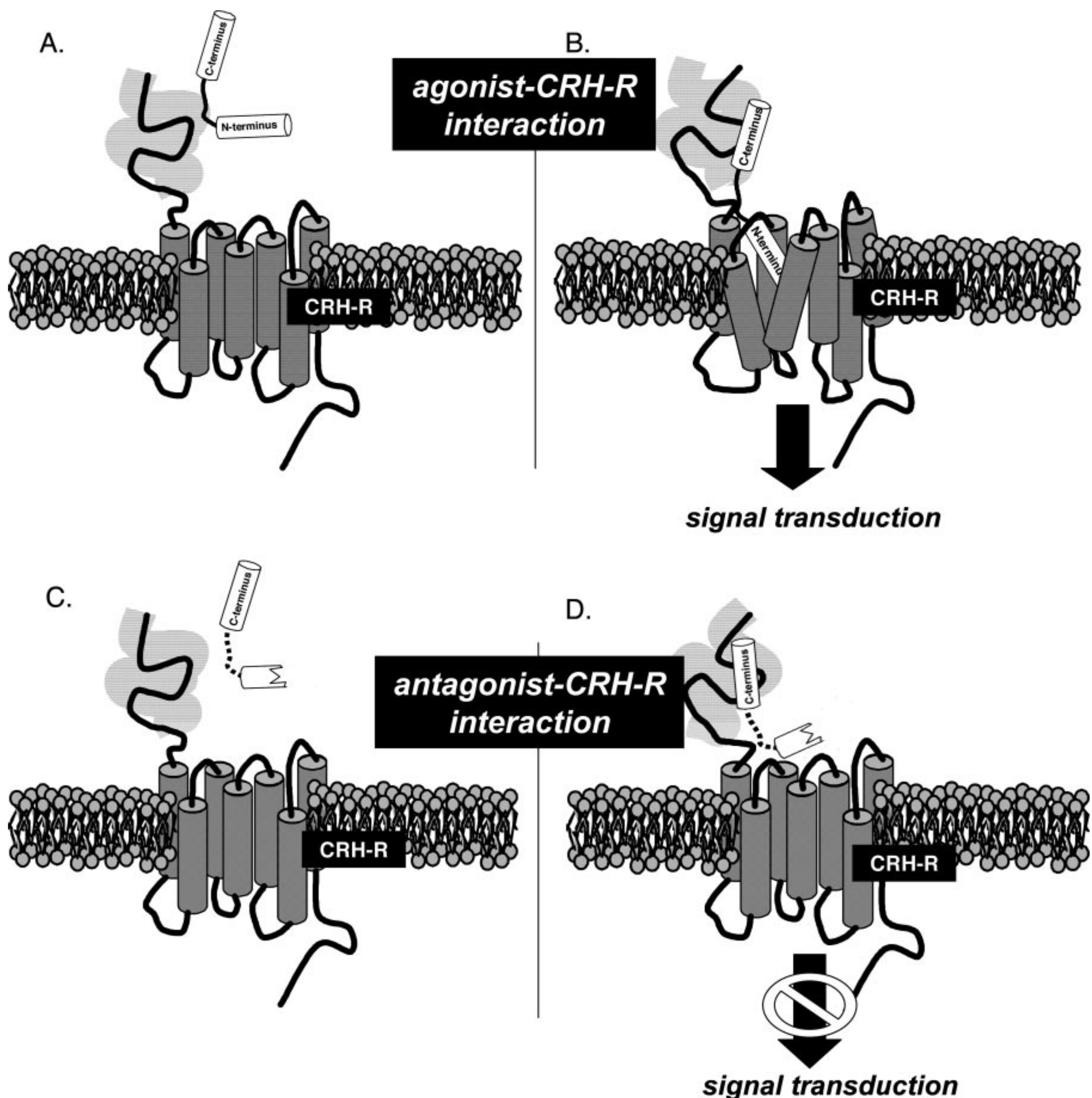

FIG. 6. Models depicting modes of action of CRH-R peptide agonists (A and B) or antagonists (C and D). According to this, CRH-R agonists recognize specific amino acid residues to the large ECD (N-domain) that enables high-affinity binding to the carboxyl-terminal regions of peptide ligands. This interaction alone is not sufficient to stimulate coupling of the receptor to G proteins, and an additional interaction is required between the receptor domain containing the transmembrane helices and intervening loops (J-domain) and the amino-terminal portion of the peptide ligand. Penetration of the agonist's N terminus into the receptor J-domain initiates intracellular signal activation. Generation of N-terminus truncated CRH peptides produces high-affinity competitive antagonists for CRH-R that cannot induce signal transduction. However, recent observations suggest that some of these antagonists (*i.e.*, astressin) can modify CRH-R important biological properties such as receptor endocytosis.

the J-domain. Unlike the CRH-R1 receptor (see below), the CRH-R2 J-domain stabilizes affinity for agonists like UCN-II by about 30-fold and might act as a CRH-R2/CRH-R1 selectivity determinant. A further weak increase in CRH-R2 affinity for UCN-II is achieved by receptor G protein coupling. Overall, the current data suggest that the J-domain of the CRH-R2 receptor binds ligands more strongly than the J-domain of the CRH-R1 (at the R state).

The location of peptide ligand binding sites on the CRH-R1 receptor has also been investigated extensively by a number of studies using mutant and chimeric mammalian CRH-R1s (93, 146, 148–152). Current data suggest a model in which: 1) agonists such as CRH bind with moderate affinity to the N-domain (residues 1–118) (100 nM); 2) interaction with the J-domain (residues 110–415) weakly stabilizes CRH binding (<2-fold); and 3) G protein binding strongly stabilizes CRH-J-domain interaction (>1000-fold). Nonpeptide antagonist affinity and the full antagonist effect are provided predominantly if not exclusively by the J-domain. The isolated N-domain binds peptide agonist ligands with affinity similar to that seen in the low-affinity, G protein-uncoupled state (R state) of the whole receptor. In contrast, the isolated J-domain

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mediates full high-affinity binding of nonpeptide antagonists and nearly full efficacy receptor activation by peptide agonists, as demonstrated by activation of the J-domain by a tethered N-terminal CRH fragment (133). A number of different regions within the N-terminal domain appear to be crucial for the binding of CRH-R1 receptor agonists and peptide antagonists (Fig. 7). Examples are the region mapping to amino acids 43–50 and a second amino acid sequence extending from position 76 to 84 of the human CRH-R1 (93). Within the latter sequence, Arg^{76} and Asn^{81}, but not Gly^{83}, appear to be important for determining receptor affinity for CRH-R1 agonists. A third amino acid cassette extending from position 68–109 of mouse CRH-R1 located in close proximity to the first TMD has also been proposed to play an important role for high-affinity agonist binding. Furthermore, agonists like CRH require interactions with the EC loops and TMD of CRH-R1 for high-affinity binding. Within the J-domain of the CRH-R1, three regions have been mapped as being important for optimal agonist binding. Two of the regions are within the second ECD, amino acids 175–178 and His^{189} at the junction of EC2 and TM3, whereas the third region is at the junction of EC3 and TM5 and involves three amino acid residues, Val^{266}, Tyr^{267}, and Thr^{268}. Also, studies in the rat CRH-R1 revealed that the third EC loop is involved in ligand binding, especially the cassette Tyr^{346}-Asn^{348} located in close proximity with 7 TMD. In contrast, the region Glu^{336}-Glu^{338} containing negatively charged amino acids of EC3, does not appear to participate in ligand binding. These studies raise the possibility that during ligand binding the polar part of this region in the third EC loop interacts directly with the polar N terminus of CRH to develop the CRH high-affinity binding site. They also suggest that different agonists require different binding domains within the receptor binding pocket; for example, although the three regions mentioned above also affect the binding of UCN-I and sauvagine, a fourth region in the EC3, Asp^{254}, has been identified to be important for sauvagine but not CRH or UCN-I binding (149).

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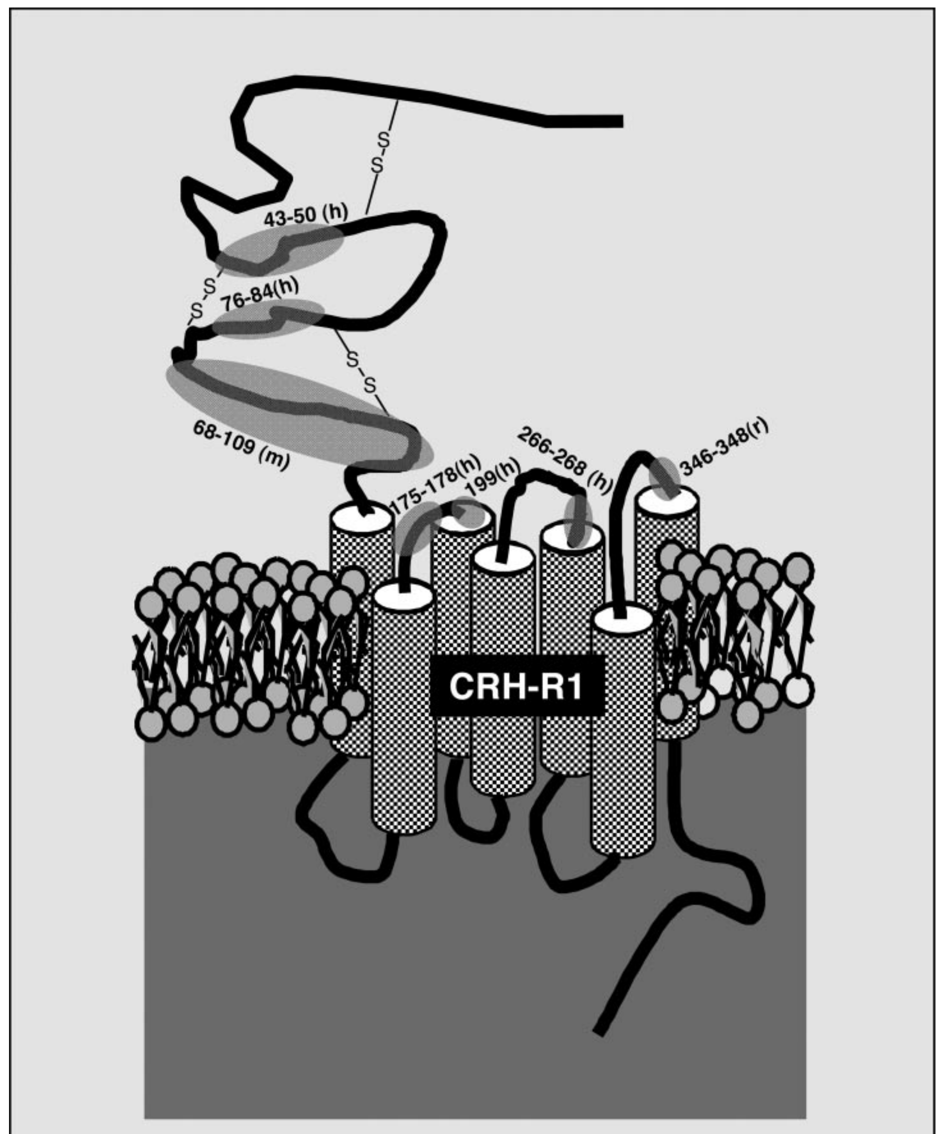

FIG. 7. Amino acid regions, present in the N terminus and EC loops, important for agonist and peptide antagonist binding to CRH-R1. A number of different regions within the N-terminal domain appear to be crucial for the binding of CRH-R1 receptor agonists and peptide antagonists. Within the amino acid sequence 76–84 of the hCRH-R1 Arg^{76}, Asn^{81} but not Gly^{83} appears to be important for determining receptor affinity for CRH-R1 agonists. Within the region at the junction of EC3/TM5, three amino acid residues appear important, Val^{266}, Tyr^{267}, and Thr^{268}. The same is true for the cassette Tyr^{346}-Asn^{348} located in close proximity with 7 TMD. These studies suggest that during ligand binding the polar part of this region in the third EC loop interacts directly with the polar N terminus of CRH to develop the CRH high-affinity binding site. *Letters in parentheses* indicate the species where CRH-R1 was obtained: r, rat; m, mouse; h, human.

C. Receptor-agonist high-affinity interaction: the role of posttranslational modifications and G proteins

The predicted CRH-R1 amino acid sequence contains multiple potential N-glycosylation sites: N38, N45, N78, N90, and N98, all of which appear to be N-glycosylated to a significant extent (154). Furthermore, this mechanism exhibits tissue-specific characteristics because the CRH-R1 is differentially glycosylated in different regions of the CNS (155). N-Glycosylation appears to be important for ligand binding, because the nonglycosylated CRH-R1 does not bind the radioligand. Although no single polysaccharide chain appears to be essential for binding, the loss of three or more polysaccharide chains significantly impairs normal ligand binding and CRH-R1 function (156).

As mentioned previously, the N termini of the B1 family of GPCRs contain multiple Cys residues forming multiple disulfide bonds (141). The CRH-R contains six conserved Cys in its N-terminal domain and one Cys in each of the first and second EC loops (Cys^{188} and Cys^{258}), respectively. Additionally, several other Cys residues are located in the TMDs (Cys^{128}, Cys^{211}, Cys^{233}, and Cys^{364}) and the first IC loop (Cys^{150}). These disulfide bonds appear to be critical for ligand recognition as shown by site-directed mutagenesis experiments (157).

The CRH-R, like all members of the GPCR family, has the intrinsic ability to couple to heterotrimeric GDP/GTP-bound proteins, G proteins, an association that stabilizes the receptor in an active high-affinity conformation. A number of studies have shown that the binding of different agonists to specific CRH-R subtypes exhibits varying degrees of sensitivity to G protein coupling. In particular, the CRH-R2 high-affinity state and binding to agonists, such as sauvagine, CRH, and UCNs, is not significantly altered by the presence or absence of receptor-G protein interaction (RG and R states, respectively) (143). In contrast, although the CRH-R1 N-domain predominantly contributes to peptide agonist affinity, the R-G coupling dramatically enhances agonist affinity via an allosteric effect (146). It has been proposed that the CRH-R1 J-domain determines R-G coupling sensitivity, and the change of agonist affinity produced by this coupling probably reflects a change in receptor conformation. Therefore, it is possible that G protein coupling produces different conformational changes within the J-domain of the CRH-R2 compared with the CRH-R1 receptor. Nonpeptide CRH-R1 antagonists, which bind the J-domain, were able to block peptide agonist binding to RG, whereas the binding of peptide antagonists, predominantly to the N-domain, was unaffected by R-G coupling. A naturally occurring model of these structural/functional relationships is the human-specific CRH-R1 variant, R1β, which has reduced binding affinity for CRH-R1 agonists although its N- and J-domains are intact (158). Impaired R-G coupling due to the presence of the 29-amino acid insert in the first IC loop probably results in reduced ligand affinity of the CRH-R1.

D. Receptor-G protein interactions: implications for signaling

Binding of an agonist to the receptor induces a conformational change and receptor activation that causes the G protein (Gα-subunit) to undergo an exchange of GDP (inactive state) for GTP (active state) (159, 160). Once GTP is bound to the Gα-subunit, it dissociates from the G$\beta\gamma$ dimer, allowing both species to activate a variety of signaling pathways. The ability of GPCRs to couple to different G protein heterotrimers, especially the Gα-subunit, is critical for activation of downstream signaling cascades and induction of diverse cellular responses. The intracellular pathway activated is largely determined by the interaction of individual GPCRs with different G proteins. Within mammalian cellular systems, more than 20 different Gα-subunits have been identified, which fall into four major classes: $G\alpha_{i/o}$, $G\alpha_s$, $G\alpha_q$, and $G\alpha_{12}$ according to sequence homologies (161). Early studies investigating the effects of CRH on pituitary ACTH release demonstrated that activation of pituitary CRH-Rs leads to a potent cAMP response through stimulation of Gs proteins (162). The structural features of Gs necessary for GPCR coupling, nucleotide binding, and adenylyl cyclase stimulation are primarily contained within the α-subunit ($G\alpha_s$), which also possesses an intrinsic GTP phosphohydrolase activity that limits the duration and strength of the signal (163).

It is now well accepted that most physiological functions of CRH in the CNS and the periphery involve CRH-R coupling to $G\alpha_s$ proteins, although other G proteins undoubtedly play a role in some functions (164). Indeed, in certain tissues such as the testis and placenta (165, 166), the effects of CRH appear to be completely $G\alpha_s$-independent, despite adequate levels of endogenous $G\alpha_s$ protein expression.

Characterization of recombinant CRH-R signaling properties in overexpression cellular systems, showed that both R1 and R2 primarily stimulate the adenylyl cyclase/cAMP pathway (45, 46) via coupling and activation of $G\alpha_s$ proteins (90), including the XLαs (extra large) protein (167), a large variant of $G\alpha_s$ protein derived from the same *GNAS1* gene that is highly expressed in neuroendocrine tissues with particularly high levels in the pituitary (168). Interestingly, these studies also demonstrated that the CRH-Rs are highly promiscuous and can activate different Gα-subunits, with an order of potency $G\alpha_s \geq G\alpha_o > G\alpha_{q/11} > G\alpha_{i1/2} > G\alpha_z$. This is not surprising and, although most GPCRs interact only with one or a small subset of G proteins, there are many examples of GPCRs that couple to multiple G proteins (169, 170), and some couple to members from all four G protein families. Similar evidence was provided by the development of a reporter strain of the fission yeast *Schizosaccharomyces pombe*, in which the pheromone-response pathways were adapted to allow ligand-dependent signaling of heterologously expressed hCRH-R1α or hCRH-R2β via either endogenous G proteins or yeast-mammalian chimeric Gα proteins (171). This approach confirmed that upon agonist activation, both CRH-R1 and -R2 can couple to multiple G proteins, including the $G\alpha_{16}$ protein. The similar profile of G protein activation by R1 and R2 is not surprising, given that the two CRH-R subtypes share regions of considerable amino acid identity, especially between the fifth and sixth TMD (third IC loop), a region that is thought to be critical for G protein coupling and signal transduction. Furthermore, studies investigating the mechanisms regulating CRH-R1-G protein interaction have shown that the CRH-R-activated G proteins can be divided into two groups, depending on their functional re-

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sponse to regulatory intracellular mechanisms such as G protein-coupled receptor kinase (GRK)-induced desensitization (homologous desensitization; see *Section VI*) (172): 1) G proteins, including $G\alpha_s$ and $G\alpha_{q/11,}$ where the efficiency of the receptor-G protein coupling is modulated by desensitization; and 2) G proteins, such as the $G\alpha_i$, where coupling efficiency is not affected by homologous desensitization.

The multiple G protein interaction characteristic of CRH-R is also evident in native tissues such as cerebral cortex, myometrium, endometrium, placenta, and adrenal (61, 86, 166, 173, 174), indicating the enormous potential of CRH to regulate diverse signaling pathways. Interestingly, the pattern of G protein activation by endogenous CRH-Rs appears to be unique for each tissue and controlled by undefined mechanisms. Due to the complex nature and individual characteristics of mammalian tissues, the precise details of the coupling of the CRH-R to their cognate G protein are as yet unknown. Also, recent evidence suggests differences in the coupling characteristics of CRH-R/G proteins between different inbred animal strain models (175); for example, investigations of the effects of CRH on hippocampal neuronal excitability revealed that hippocampal CRH-Rs can activate $G\alpha_s$, $G\alpha_{q/11}$, and $G\alpha_i$ in C57BL/6N mice, and only $G\alpha_{q/11}$ in BALB/c mice, suggesting genetic background influences on receptor/G protein coupling.

As mentioned previously, different agonists interact with specific binding domains within the receptor binding pocket, and this would probably lead to distinct active conformations of the CRH-R and different profiles of G protein activation. *In vitro* experiments using yeast reporter strains suggest that the agonist plays a major role in determining the G protein activation potency and profile for each CRH-R (171). Also, in mammalian native cells endogenously expressing CRH-R and cellular overexpression systems, CRH and UCN-I exhibit significant differences in the activation of certain G proteins and altered signaling properties (176, 177). For example, in HEK293 cells overexpressing hCRH-R1α and human smooth muscle cells, UCN-I-induced $G\alpha_q$-protein activation is significantly more potent compared with CRH, resulting in agonist-specific stimulation of extracellular signal regulated protein kinase (ERK1/2) cascade (177). These findings confirmed the critical role of CRH-R agonists as determinants of G protein coupling and signaling, in accordance with the "agonist-dependent signaling trafficking" hypothesis (178, 179). Other studies in native tissues demonstrated that the CRH-R-G protein coupling is not "static" but is rather dynamically regulated by physiological processes. For example, progression of human pregnancy toward term is associated with changes in myometrial smooth muscle tissue G protein coupled to CRH-R, and CRH-induced $G\alpha_q$-protein activation was evident at 39 wk but not at 33 wk gestation (176) (Fig. 8). This appears to be an important feature of the CRH-R that allows agonists such as CRH to activate different signaling cascades during the different stages of pregnancy and labor and thus play an important role in the transition of the human uterus from a state of relaxation to one of increased contractility and active labor (176). Therefore, it appears that a revised hypothesis, which should include the agonist, receptor subtype, and cell type as the critical determinants of "signaling trafficking", would more accurately represent the CRH-R/G protein interactions and downstream signaling pathways activation.

There is little information regarding the CRH-R structural domains important for G protein interaction. Studies on the signaling properties of the CRH-R1 variants suggest that an intact conformation and/or orientation of the C-tail is crucial

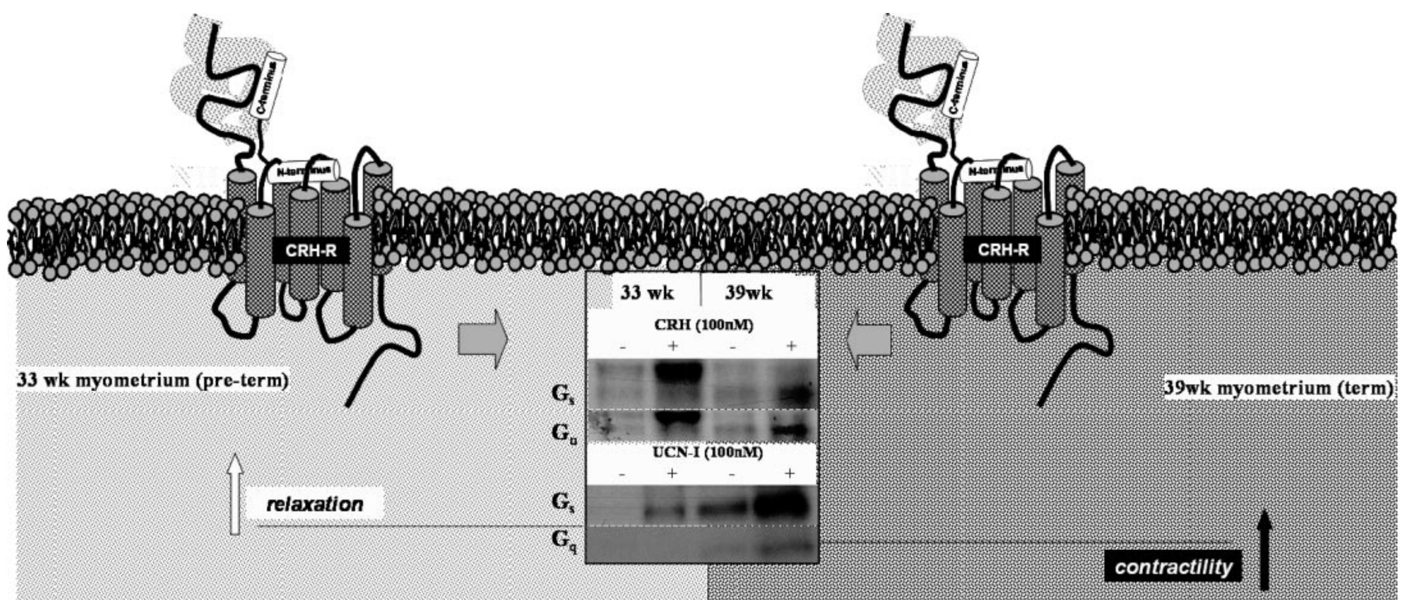

FIG. 8. The human myometrium during pregnancy as an example of a physiological process dynamically regulating CRH-R-G protein coupling. Progression of human pregnancy toward term is associated with changes in myometrial smooth muscle tissue G protein coupled to CRH-R (shown in the *inset* by GTP-AA photoaffinity labeling of activated G proteins in human pregnant myometrium at wk 33 or 39 of pregnancy before the onset of labor). Although the mechanisms involved in this dynamic regulation are unknown, this appears to be an important feature of the CRH-R that allows agonists such as CRH to activate different signaling cascades during the different stages of pregnancy and labor, and thus play an important role in the transition of the human uterus from a state of relaxation to one of increased contractility and active labor.

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for receptor interaction with G proteins, and receptor variants such as the CRH-R1d with a distorted C-tail exhibit profound impairment in G protein activation (90). For many GPCRs, the second and third IC loops and the proximal part of the C-terminal tail can be directly involved in G protein-receptor interaction. Junctions between TMD3/IC2, TMD5/IC3, and IC3/TMD6 share conserved hydrophobic residues, and it is assumed form a binding pocket allowing the recruitment of G protein to the receptor. Charged amino acids in the proximal and distal regions of IC3 are involved in the recognition and direct interaction with a specific G protein. Amino acids within the TMD3/IC2 junction would then act as a switch enabling G protein activation (180). The CRH-R1/G protein coupling appears to involve similar interactions because preliminary evidence from our laboratory suggests that specific amino acid cassettes proximal to the TMD5/IC3 and IC3/TMD6 junctions appear to participate in the active conformation of the CRH-R1α and specific recognition of the Gα_s protein following agonist binding (181). Furthermore, other amino acids within IC3 appear to be important for efficient receptor-G protein interaction. We identified Ser^{301} as an important residue for interaction with Gα_s, Gα_o, G$\alpha_{q/11}$, and G$\alpha_{i1/2}$ (182). Studies using mutant receptors showed that the nature of the residue present in position 301 plays a significant role in determining the G protein activation efficiency of the hCRH-R1, either through stabilization of the amphipathic α-helix of the IC3 or direct amino acid to amino acid interactions between CRH-R1α and G proteins. Gα_o proteins appeared to be the most sensitive to amino acid substitution at this position, whereas the weakly activated Gα_i was the least. These studies also demonstrated the preference of Gα_s protein for acidic, but not charged, amino acid residues at position 301 to achieve full activation through the interaction with the receptor.

Another important aspect of CRH-R interactions with G proteins is the intracellular mechanisms regulating its ability of the CRH-R1 to switch between different G proteins and signaling cascades. There is very little evidence relating to this, however, but it is conceivable that tissue-specific mechanisms are involved, based on the unique profiles of activated G proteins seen in various tissues (61, 86, 164, 173, 174). Evidence from other GPCRs suggests that receptor phosphorylation by intracellular protein kinases might be one such important mechanism. Phosphorylation by PKA of the β2-adrenergic receptor switches its coupling from Gα_s to Gα_i (183). A similar self-regulatory mechanism regulates CRH-R1α coupling to G proteins, and Gα_s-induced PKA activation results in CRH-R1α phosphorylation and selective impairment of CRH-R1α/Gα_q protein coupling, suggesting that phosphorylation of Ser^{301} may prevent optimal interaction of the CRH-R1α with specific G proteins, such as Gα_q protein (182).

V. CRH-R Signaling Characteristics

A. Activation of the cAMP/PKA signaling pathway

In most cells, CRH and CRH-like agonists exert various physiological effects via activation of the adenylyl cyclase-cAMP signaling pathway, resulting from the potent activation of Gα_s protein. This pathway initiates intracellular events both in the cytoplasm, resulting in the acute post-translational modification of target proteins by PKA, and in the nucleus at the level of gene transcription regulation by cAMP response element-binding (CREB) proteins (184, 185). Pharmacological agents have proved useful in dissecting the contribution of the cAMP/PKA/CREB signaling pathway in mediating the biological actions of CRH and the UCNs in different systems (85, 164, 187–196) (Table 1). A further level of complexity is provided by accumulating evidence that implicates other signaling molecules in the amplification or modification of these cAMP/PKA-mediated responses. Examples of this include activation of membrane-bound guanylyl cyclase activity and increased cGMP production in smooth muscle cells (174), stimulation of the nuclear factor-κB pathway in mouse thymocytes (198), phosphorylation of glycogen synthase kinase-3 in CNS neurons (199), suppression of the activity of slow Ca^{2+}-activated K^+ current [I(sAHP)] in CA1 hippocampal pyramidal neurons (200), and increased tyrosine hydroxylase phosphorylation in rat adrenal PC12 cells (201).

CRH-induced phosphorylation and activation of CREB leads to downstream regulation of genes containing the Ca^{2+}/cAMP response element (202). Examples of such genes include *c-fos* (203), macrophage migration-inhibitory factor gene *Mif* (204), and orphan nuclear receptors *Nur77* and *Nur1* (205). Activation of the latter appears to involve multiple signaling cascades as revealed in detailed studies using molecular and pharmacological inhibitors in AtT-20 cells (206).

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TABLE 1. Examples of CRH and CRH-related peptide biological effects mediated through activation of the cAMP/PKA/CREB pathway

Biological actions of CRH and/or UCN-I involving cAMP	CRH-R subtype involved	Ref.
Release of ACTH in the pituitary	CRH-R1	164
Regulation of cytokine and vascular endothelial growth factor secretion in mast cells	CRH-R1	85
Up-regulation of brain-derived neurotrophic factor in cerebellar neurons	CRH-R1	187
Brain region-specific neuroprotection against amyloid β 25–35 and glutamate toxicity	CRH-R1	188, 189
Activation of the inflammatory response in synovial tissue endothelium through the NURR subfamily of nuclear orphan receptors	CRH-R1	190
Antiproliferative effects in endometrial adenocarcinoma cells	CRH-R1	191
Stimulation of progesterone production and steroidogenic acute regulatory protein expression in Leydig tumor cells	CRH-R1 (?)	192
UCN-I-induced vasodilatation of aortas and renal arteries	CRH-R2	193, 194
CRH and UCN-I stimulation of atrial natriuretic peptide and brain natriuretic peptide from cardiomyocytes during cardiac hypertrophy	CRH-R2	195, 196

?, Not certain.

These showed that activation of cAMP by CRH induces *Nur77* and *Nurr1* mRNA expression and transcription at the NurRE site leading to transcriptional activation of POMC expression. The molecules involved in this process include PKA, calcium/calmodulin kinase II (for Nur induction/activation), and MAPK (for *Nur77* phosphorylation-activation).

Few studies have investigated the transcriptional response of CRH-R1 signaling at the level of the genome. One report (207) using oligonucleotide microarrays in At-20 corticotroph cells, identified many novel CRH/CRH-R1, and potentially cAMP, responsive genes including transcription factors such as the cAMP-responsive element modulator, nuclear factor regulated by IL-3 and Jun-B, secreted peptides such as choleystokinin, and signaling modulating proteins such as receptor (calcitonin) activity modifying protein 3, cAMP-specific phosphodiesterase 4B, the regulatory subunit phosphatidylinositol 3-kinase p85, and regulator of G protein signaling 2 that potentially can attenuate CRH signaling through negative feedback interactions. Also, in a more focused approach, Sirianni *et al.* (208, 209) employed oligonucleotide microarrays in human fetal zone and in definitive/transitional zone adrenal cells and demonstrated that CRH and UCN-I, acting via the CRH-R1α receptor, can directly induce the expression of various enzymes involved in adrenal dehydroepiandrosterone sulfate and cortisol production. These include steroidogenic acute regulatory protein, cholesterol side chain cleavage (CYP11A), 17α-hydroxylase (CYP17), 21-hydroxylase (CYP21), dehydroepiandrosterone sulfotransferase (SULT2A1), 3β-hydroxysteroid dehydrogenase type II (HSD3B2), 11β-hydroxylase (CYP11B1). Significantly, both studies in the corticotrophs and adrenal cells also demonstrated the ability of CRH to up-regulate the expression of CRH-R1 gene (207, 208).

B. cAMP-independent signaling pathways: CRH-Rs and activation of MAPKs

In some studies, pharmacological inhibition of the cAMP/PKA pathway failed to abolish the biological effects of CRH and CRH-related agonists, suggesting that these agonists and their receptors can induce cellular events through alternative signaling pathways. An example of the latter is the tissue-specific regulation of the nuclear factor-κB pathway in human epidermal keratinocytes and pituitary corticotroph cells where it is involved in the activation of the POMC gene (210, 211). It is now well established that the CRH-Rs can activate a plethora of intracellular pathways both *in vivo* and *in vitro*, in agreement with the data on multiple G protein activation. These include PKC, PKB/Akt, ERK, and p38 MAPK, as well as other important signaling molecules, such as Ca^{2+}, nitric oxide synthase (NOS), guanylyl cyclase, prostaglandins, RhoA/ Rho kinase, Fas and Fas ligand (212–219).

A number of signaling molecules mediating the biological functions of CRH-Rs in the CNS and the periphery have attracted particular attention, especially the family of MAPK enzymes, in particular ERK and p38 MAPK. Both enzymes are stimulated by agonist-activated CRH-R1 or CRH-R2 in cells expressing either endogenous or recombinant CRH-Rs (76, 177, 202, 206, 212, 219–221). This signaling pathway appears to mediate important physiological functions of CRH and CRH-related agonists such as cardioprotection against ischemia reperfusion injury (76), the behavioral and memory adaptation to stress (222), neuroprotection (189, 223), vasodilatation (224), and smooth muscle contractility (219). Furthermore, ERK mediates the CRH-induced activation of Nur transcription factors and induction of POMC in AtT-20 cells (206) and the CRH-induced suppression of caspase-dependent apoptosis and cytotoxicity in Y79 retinoblastoma cells (225), whereas p38 MAPK is involved in CRH inhibitory effects on IL-18 expression in human keratinocytes (226). Interestingly, studies on the G proteins and signaling molecules involved downstream of CRH-Rs suggest unique tissue-specific features. For example, in brain and neuronal cells such as AtT-20 or Y79 neuroblastoma cells, agonist-induced activation of CRH-Rs and ERK phosphorylation involves the Gα_s protein-adenylyl cyclase pathway (206, 225), whereas in uterine smooth muscle cells this effect appears to be exerted primarily, but not exclusively, via activation of the Gα_q-IP$_3$-PKC pathway (177). In uterine smooth muscle, the intracellular signaling pathways appear quite complex because activation of the Gα_s protein-adenylyl cyclase-PKA pathway attenuates agonist-induced ERK activation through PKA-induced phosphorylation of the CRH-R1 at position Ser^{301}, a posttranslational modification event that inhibits maximal coupling of CRH-R with Gα_q protein (182) and allows a CRH-R1 signaling switch. These events are likely to have significant physiological significance, because ERK activation plays a major role in cell proliferation, and the cross-talk between the adenylyl cyclase/PKA and the ERK cascades involving PKA-mediated regulation of various downstream intracellular molecules is well established (227, 228). For example, CRH activation of the CRH-R1-adenylyl cyclase-PKA pathway in the Ishikawa human endometrial adenocarcinoma cell line has been shown to inhibit cell growth and proliferation (191). Although not examined in this study, it is conceivable that this effect is mediated via inhibition of ERK activity. Another example of the potential physiological importance of CRH-R1 signal switching is the human pregnant myometrium. At term, UCN-I induces ERK activation and possibly enhances myometrial contractility (177, 229), because ERK has been proposed to be involved in the regulation of myometrial contractility in rats (230). On the other hand, human pregnancy is associated with increased myometrial PKA activity (40), which might provide a mechanism for preventing "inappropriate" ERK activation by CRH-like agonists to maintain uterine quiescence before the timely onset of labor. This view is supported by our studies of CRH-R-G protein coupling during different stages of pregnancy, which have shown activation of Gq proteins by UCN in term (39 wk gestation) but not preterm (33 wk gestation), myometrial tissue (Fig. 8), coincidental with a reduction in the ability of CRH-R agonists to activate Gα_s protein (176).

The unique tissue-specific requirement for particular G protein activation to stimulate ERK activity is closely determined by the ability of individual CRH-R agonists to activate the necessary G proteins. Our own studies have shown that in native myometrial cells UCN-I, but not CRH, can trigger ERK activation because only UCN-I induces CRH-R-Gα_q protein coupling and translocation of PKC with sufficient

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potency to activate this pathway (177). In contrast, in brain tissue, where ERK phosphorylation and activation are primarily dependent on $G\alpha_s$ protein-PKA activation, both CRH and UCN-I are capable of activating this cascade (189). This finding further supports the hypothesis of agonist, receptor subtype, and cell type determinants of signaling trafficking.

Intriguingly, the CRH-R-ERK interaction appears to be extremely versatile and depends upon activation of multiple signaling pathways, a common feature of many GPCRs that regulate ERK activity. This probably reflects the crucial role of this signaling cascade in mediating the biological effects of CRH in different cellular systems. In addition to the $G\alpha_q$-IP_3-PKC pathway, other signaling molecules such as $G\alpha_i$ and $G\alpha_o$ proteins, phosphatidylinositol-3-OH kinase, MAPK kinase 1, Raf-1 kinase, tyrosine kinases, and possibly intracellular Ca^{2+} have all been implicated in CRH-R-induced ERK activation (220), highlighting the complexity of these interactions.

C. CRH-Rs and the regulation of the NO/cGMP signaling pathway

Another important signaling pathway that is regulated by CRH and CRH-related agonists in various physiological systems is the nitric oxide (NO)/cGMP pathway, a major signaling cascade in the control of vascular tone (231). In a number of different cellular systems, CRH and CRH-related peptides can modulate the expression and activity of NOS, the enzyme that is primarily responsible for intracellular formation of NO, and the induction of intracellular events involving activation of the soluble form of guanylyl cyclase and production of the second messenger cGMP. In uterine smooth muscle cells, activation of CRH-R1 leads to up-regulation of the constitutive (endothelial and brain), but not inducible, NOS through a $G\alpha_s$ protein-PKA independent pathway (174). Similar CRH-R1-mediated interactions have been observed in human placenta (128). In this tissue, however, this effect is mediated via CRH-R2 specific agonists such as UCN II, possibly reflecting differences in the relative expression levels/accessibility between the CRH-R subtypes in the two cellular systems. This placental signaling pathway appears to play a major role in the CRH-induced control of vascular tone (232), and abnormal down-regulation of placental CRH-R expression has been implicated in the increased vascular resistance of this organ in women with preeclampsia (127, 128). Interestingly, in other cellular systems, CRH differentially regulates indicible NOS (iNOS) expression, suggesting a more complex relationship and highlighting the tissue-specific effects of CRH-R agonists. Examples are H5V murine endothelioma cells, which express both CRH-R subtypes and in which CRH attenuates cytokine-stimulated iNOS protein expression, an effect mediated via CRH-R1 receptors, and HUVEC cells that express only CRH-R2 and in which CRH potentiates cytokine-induced iNOS expression (214).

CRH and CRH-like agonists can also acutely augment NOS and soluble guanylyl cyclase (sGC) activity in a NO-independent pathway, resulting in increased intracellular levels of cGMP (128, 174). This pathway also appears to be important in CRH and UCN-induced relaxation of placental vasculature and peripheral arteries, a process that involves release of NO with subsequent activation of Ca^{2+}-activated K^+ channels in vascular smooth muscle cells via a cGMP-dependent mechanism (224, 234). Acute regulation of NO and/or sGC/cGMP signaling pathways has been described in the pituitary involving not just CRH, but a number of adenylyl cyclase-activating agonists (217). These agonists stimulate cGMP production through PKA-induced phosphorylation of Ser^{107}-Ser^{108} N-terminal residues of the α_1-sGC subunit that most likely stabilizes the $NO/\alpha_1\beta_1$ sGC heterodimer complex (236). Furthermore, in uterine smooth muscle cells, CRH can also potentiate intracellular cGMP production through a distinct pathway involving enhancement of the membrane-bound form of guanylyl cyclase activity via a partially PKA-dependent mechanism (128).

VI. Regulation of CRH-R Functional Activity

The physiological effects of CRH and CRH-related agonists in target tissues depend on sufficient expression of functional CRH-Rs. As described previously, alterations in the expression or the splicing pattern of the CRH-R gene can influence the relative potency of an agonist. At present there is insufficient information concerning the cellular mechanisms that control CRH-R activity in target cells. It is known, however, that GPCR signaling can be rapidly attenuated by receptor phosphorylation involving protein kinases such as PKA, PKC, and the GRKs that allow interaction with arrestins leading to receptor desensitization and uncoupling from G proteins (237) with subsequent receptor internalization (238). There are a number of physiological examples consistent with the notion that high levels of CRH can desensitize the CRH-R1 signaling response, in particular the adenylyl cyclase activation. These have been described in the rat pituitary, human myometrial smooth muscle, mouse fibroblasts, human retinoblastoma and neuroblastoma cell lines Y79 and IMR-32, respectively (239–243), as well as in cell lines overexpressing recombinant CRH-R1 (240, 244), albeit the kinetics of signal desensitization and recovery are quite different.

Most studies agree that homologous desensitization of CRH-R1 is a PKA-independent process (240, 244), despite the fact that the CRH-R1 sequence contains a putative PKA phosphorylation site at position Ser^{301} (40). Upon activation by the $G\alpha_s$ protein-cAMP pathway, PKA can indeed phosphorylate the CRH-R1, and this modification appears to be important for specific G protein coupling and signaling selectivity (182). The same might be true for the CRH-R2 because both CRH-R subtypes have identical potential PKA phosphorylation sites. Studies in different cellular systems (HEK293 and Y79 neuroblastoma cells) have also identified multiple GRKs involved in CRH-R1-phosphorylation and homologous desensitization: GRK3 appears to be the major "cytosolic" GRK isoform, and GRK6 the main "membrane-bound" GRK isoform. GRK3 requires $G\beta\gamma$-subunits for its recruitment to the plasma membrane and association with the CRH-R1; by contrast, GRK6 is able to interact with the CRH-R1 in a $G\beta\gamma$-subunit-independent manner, due to its constitutive association with the plasma membrane (245). However, recent

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studies suggest that in cellular systems where GRK3 is not present (such as AtT-20 corticotroph cells), GRK2 participates in homologous CRH-R1 desensitization (246). The cAMP/PKA pathway may also have a role in this mechanism by activating GRK2 through phosphorylation. Putative sites of GRK-mediated phosphorylation of CRH-R1 have been searched within the distal portion of the cytoplasmic tail of the CRH-R1, which contains a cluster of Ser/Thr residues (Ser^{396}-Ser^{405}). Site-directed mutagenesis studies identified Thr^{399} as a GRK-phospho-acceptor residue (240) important for mediating CRH-R1 homologous desensitization. Interestingly, in some biological systems such as the human retinoblastoma Y79 cells, the CRH-R1 appears to control its own desensitization rate by up-regulating GRK3 mRNA levels (235). This effect appears to be directed specifically toward GRK3, because GRK2 levels are not affected, and during prolonged exposure to high CRH it might act as a cellular negative feedback mechanism to maximize CRH-R1 desensitization. This phenomenon also appears to be agonist-dependent because in the same cellular setting only CRH, but not UCN-I, induced GRK3 up-regulation.

Most studies investigating the role of homologous desensitization on CRH-R signaling have focused on the activation of adenylyl cyclase, although the CRH-R can potentially activate multiple signaling cascades, in line with the concept of GPCR signaling diversity as a result of different ternary complexes and active conformational states of the receptor (233). Preliminary studies in a CRH-R1 overexpression cellular system (172) suggest that homologous desensitization primarily attenuates $G\alpha_s$ and $G\alpha_{q/11}$-driven signaling cascades. In contrast, CRH-R1 receptor states that induce $G\alpha_i$ protein activation are not susceptible to desensitization, either because the important Ser/Thr are not accessible for phosphorylation or potential phosphorylation does not interfere with the $G\alpha_i$ protein coupling. This might represent a regulatory mechanism that allows a more rapid decline of some CRH-R1 stimulatory effects.

GRK-induced phosphorylation of the agonist-activated receptor at specific Ser/Thr residues interferes with the G protein coupling of the GPCRs and facilitates the interaction of the receptor with intracellular proteins that maintain the inactive state of the receptor and favor its internalization (238). For many but not all GPCRs, this process involves receptor interaction with members of the arrestin family of proteins (197). The binary complex formed between the phosphorylated GPCR and arrestin initiates two important intracellular processes, receptor uncoupling and internalization, that are involved in both the desensitization and the recovery of cellular responses. In HEK293 cells overexpressing CRH-R1 receptors, β-arrestin appears to be involved in CRH-R1 desensitization and internalization (240). Once the receptor is activated by agonist binding, the β-arrestin rapidly translocates to the plasma membrane in close proximity to the CRH-R1. Phosphorylation of the CRH-R1 by GRK and intact phosphorylation sites within the IC3 and the C terminus are required for efficient β-arrestin recruitment (186). Although the internalization characteristics of the CRH-R1 have not yet been characterized in detail, some preliminary evidence suggests the involvement of clathrin-coated pits in CRH-R1 internalization (153, 186). However, under different experimental conditions, CRH-R1 internalization appears to be independent of the degree of β-arrestin recruitment and/or receptor phosphorylation (186), revealing a potential versatility of the CRH-R1 internalization mechanisms. Furthermore, recent data suggest a more complex relationship between agonist-CRH-R activation-signal generation and CRH-R1 internalization. Astressin, an amino-terminal truncated analog of CRH with high-affinity binding to the N terminus of the CRH-R1, which is thought to act as a neutral competitive antagonist, is capable of inducing CRH-R1 internalization, despite being unable to stimulate CRH-R1 signaling (153). This finding might be of considerable significance for the design of CRH-R antagonists for specific disorders because it would allow suppression of receptor signaling by inducing its internalization. Intriguingly, CRH and astressin employ distinct internalization pathways. Binding of CRH to CRH-R1 induces phosphorylation, β-arrestin recruitment, and receptor internalization most likely through clathrin-coated pits, whereas binding of astressin fails to induce receptor phosphorylation or β-arrestin recruitment. Instead, receptor endocytosis occurs through a pathway involving sequestration into intracellular compartments other than clathrin-coated pits and caveolae. To induce CRH-R1 internalization, astressin must interact with both the N terminus and the J-domain of the receptor. These data raise the possibility that in the CRH-R1 the two processes of receptor activation and signaling and receptor internalization might not be closely related and demonstrate that under certain conditions (*e.g.*, antagonist binding), the CRH-R1 can adopt distinct active conformations important for specific functions such as receptor internalization.

The potential role of PKC has also been investigated in CRH-R homologous and heterologous desensitization and internalization. Most studies agree that PKC activation is not involved in homologous CRH-R1 desensitization and internalization (186), although in some cellular systems, CRH actions are modulated by PKC, and accumulating evidence suggests that the CRH-R1 is indeed a target of PKC-induced phosphorylation. For example, in Y79 human neuroblastoma cells, PKC is involved in the heterologous, but not the homologous, desensitization of the CRH-induced cAMP response (147). Furthermore, oxytocin-induced PKC activation in human pregnant myometrium at term inhibits CRH-R activity (91). The latter experimental paradigm provided evidence that some, but not all, CRH-R isoforms are sensitive to oxytocin-induced PKC activation in human term myometrium. Data from our laboratory suggest that only the human-specific CRH-R1β variant appears to be susceptible to PKC-induced desensitization resulting in the endocytosis of the CRH-R1β variant in a β-arrestin independent manner (D. Markovic, N. Papadopoulou, T. Teli, H. Randeva, M. A. Levine, E. W. Hillhouse, D. K. Grammatopoulos, submitted for publication). Furthermore, the presence of Ser^{408} in the distal part of the CRH-R1α C-tail appears to confer its resistance to PKC-induced desensitization and internalization because replacement of Ser^{408} rendered the CRH-R1α susceptible to PKC-induced desensitization and internalization. Variable usage of specific phospho-acceptor sites by protein kinases is a phenomenon described for other GPCRs (75). It is possible that PKC initially targets Ser^{408} as the primary

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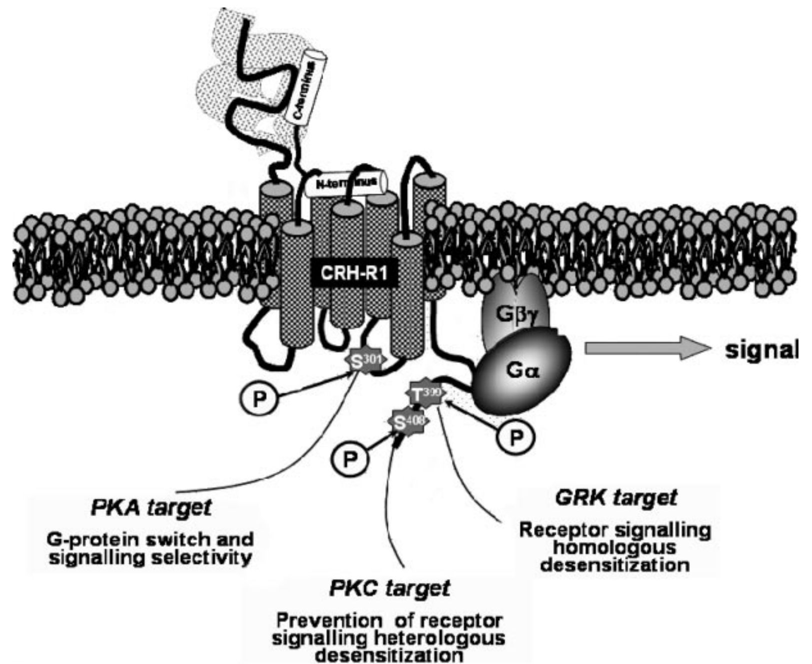

FIG. 9. Amino acid residues, present in the third IC loop and carboxy-terminus, important for regulation of CRH-R1 biological activity by Ser/Thr protein kinases. CRH-R phosphorylation by second messenger-activated and GRKs appears to be an important mechanism for modulating receptor G protein-coupling efficiency, signaling potency and cross-talk between distinct signaling cascades (PKA-induced phosphorylation at position Ser^{301}) and heterologous regulation of CRH-R1 activity (PKC-induced phosphorylation at position Ser^{408}). Furthermore, GRK-induced phosphorylation at specific residues in the carboxy-terminus of CRH-R1 (Thr^{399}) is critical for receptor β-arrestin-dependent desensitization leading to receptor endocytosis and signal termination.

phospho-acceptor site and phosphorylation of this residue prevents receptor desensitization and internalization. Lack of this residue may lead PKC to target other putative phosphorylation sites and initiate receptor desensitization and internalization, directly or indirectly via *trans*-activation of specific GRK isoforms or other protein-protein interactions. This might represent an important mechanism for functional regulation of CRH signaling in target cells. An overview of these intracellular mechanisms targeting the CRH-R1 is presented in Fig. 9.

VII. Conclusions

Like many other GPCRs, CRH-Rs are extremely versatile transduction molecules exerting many different biological actions in various tissues. Their biological activity is governed by a diverse array of mechanisms involving distinct genes regulated by one or more promoters, complex mRNA splicing mechanisms which are poorly characterized, and many tissue-specific interactions with other signaling molecules. CRH-Rs play a crucial role in mammalian physiology, and additional research is required to unravel the particular characteristics of this complex picture. The elucidation of the molecular basis of CRH-R signaling regulation and its tissue-specific determinants is proving extremely useful in understanding the role of CRH and CRH-like agonists in mammalian pathophysiology, which might aid in the development of more specific therapeutic approaches.

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E.W.H. and D.K.G. have nothing to declare.

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