THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 287, NO. 12, pp. 8974–8985, March 16, 2012
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Mapping Structural Determinants within Third Intracellular Loop That Direct Signaling Specificity of Type 1 Corticotropin-releasing Hormone Receptor*[S]

Received for publication, June 22, 2011, and in revised form, January 13, 2012 Published, JBC Papers in Press, January 13, 2012, DOI 10.1074/jbc.M111.272161

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Background: The CRH-R1 is key for mammalian adaptation to stress; however, the structural determinants of signal transduction are unknown.
Results: Amino acids identified within CRH-R1 IC3 appear crucial for G protein-dependent cAMP and ERK1/2 signaling.
Conclusion: These microdomains provide a tight control of CRH-R1 differential coupling to distinct G proteins and downstream effectors.
Significance: This might prevent hyperstimulation of stress-induced responses.

The type 1 corticotropin-releasing hormone receptor (CRH-R1) influences biological responses important for adaptation to stressful stimuli, through activation of multiple downstream effectors. The structural motifs within CRH-R1 that mediate G protein activation and signaling selectivity are unknown. The aim of this study was to gain insights about important structural determinants within the third intracellular loop (IC3) of the human CRH-R1α important for cAMP and ERK1/2 pathways activation and selectivity. We investigated the role of the juxtamembrane regions of IC3 by mutating amino acid cassettes or specific residues to alanine. Although simultaneous tandem alanine mutations of both juxtamembrane regions Arg^{292}-Met^{295} and Lys^{311}-Lys^{314} reduced ligand binding and impaired signaling, all other mutant receptors retained high affinity binding, indistinguishable from wild-type receptor. Agonist-activated receptors with tandem mutations at the proximal or distal terminal segments enhanced activation of adenylyl cyclase by 50–75% and diminished activation of inositol trisphosphate and ERK1/2 by 60–80%. Single Ala mutations identified Arg^{292}, Lys^{297}, Arg^{310}, Lys^{311}, and Lys^{314} as important residues for the enhanced activation of adenylyl cyclase, partly due to reduced inhibition of adenylyl cyclase activity by pertussis toxin-sensitive G proteins. In contrast, mutation of Arg^{299} reduced receptor signaling activity and cAMP response. Basic as well as aliphatic amino acids within both juxtamembrane regions were identified as important for ERK1/2 phosphorylation through activation of pertussis toxin-sensitive G proteins as well as G_q proteins. These data uncovered unexpected roles for key amino acids within the highly conserved hydrophobic N- and C-terminal microdomains of IC3 in the coordination of CRH-R1 signaling activity.

The type 1 corticotropin-releasing hormone receptor (CRH-R1)[3] belongs to the class B1 subfamily of heptahelical receptors ("brain-gut" neuropeptide receptors) that transduce signals primarily, but not exclusively, through activation of G proteins (*i.e.* G protein-coupled receptors, GPCRs). The CRH-R1 is widely expressed in the brain and peripheral tissues and orchestrates mammalian homeostatic adaptation mechanisms to stressful stimuli (1). The CRH-R1 elicits a wide range of biological effects, sometimes in a tissue-specific manner, via activation of a variety of signaling pathways using diverse G proteins (2). A number of downstream signal effectors for CRH-R1 have been described; however, the cAMP and the extracellular signal-regulated kinase (ERK)1/2 pathways represent the principal signals that mediate CRH-R1 action after interaction with agonists such as CRH and urocortin 1 (Ucn1) in target cells (3, 4).

A variety of molecular mechanisms modify CRH-R1 signaling. Previous studies have shown that protein kinases such as PKA mediate cross-talk between signaling pathways via phosphorylation of specific amino acid residues in the third intracellular loop (IC3) of CRH-R1, thereby regulating efficiency of receptor coupling to G_q protein and downstream ERK1/2 signaling (5). G protein-coupled receptor kinases also influence CRH-R1 action through amino acid phosphorylation that leads

* This work was supported by a Wellcome Trust University Award (to D. K. G.). Dedicated to Wylie Vale and his enormous legacy.
⌘ *Author's Choice*—Final version full access.
[S] This article contains supplemental Tables 1–3 and Figs. 1–3.
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[3] The abbreviations used are: CRH-R1, type 1 corticotropin-releasing hormone receptor; AC, adenylyl cyclase; DN, dominant negative; GPCR, G protein-coupled receptor; IC3, third intracellular loop; IP_3, inositol trisphosphate; NF023, 8,8′-(carbonylbis(imino-3,1-phenylene carbonylimino))-bis(1,3,5-naphthalenetrisulfonic acid); PTX, pertussis toxin; TMD, transmembrane domain; Ucn1, urocortin 1.

to desensitization and subsequent endocytosis of receptor endocytosis (6), a mechanism that can either reduce specific signaling cascades such as the adenylyl cyclase (AC)/cAMP pathway or activate ERK1/2 and p38MAPK cascades (7). Specific Ser/Thr phosphoacceptor residues have been identified in both the IC3 and the distal segment of the C terminus of CRH-R1 (6, 8). Phosphorylation of the latter domain, especially at position Thr^{399}, appears to be important for β-arrestin2 recruitment and desensitization of cAMP signaling (6). Interestingly, mechanisms leading to desensitization do not affect coupling of CRH-R1 to all classes of G proteins (9). Receptor conformations that activate G_s and $G_q/_{11}$ proteins are sensitive to desensitization, whereas G_i proteins are activated by different receptor states that do not promote signaling desensitization.

The domains that determine coupling of CRH-R1 coupling to distinct G proteins and downstream signaling pathways are not well understood. Most GPCR dynamic functions such as receptor activation, internalization, desensitization, and resensitization involve key structural determinants present in the cytoplasmic loops and carboxyl tail (10–13). However, the extensive structural diversity among GPCRs suggests that efficient G protein coupling can be achieved through various unrelated structural motifs. In fact, conformation and charge rather than primary sequence appear to be the major determinants for G protein activation. Past studies have identified the IC3 as a key element that regulates the ability of GPCRs to activate G proteins and trigger intracellular responses (14, 15). Within IC3, the juxtamembrane regions of IC3 that form cationic α-helical structures (16) appear to be important for the activation of distinct unrelated G protein subtypes. Moreover, the IC3 appears to be important for G protein-independent signaling of GPCRs; for example the IC3 N-terminal part of CXCR4 is required for JAK2 activation leading to phosphorylation of only one cytoplasmic Tyr residue, present at the C terminus of IC2, which triggers STAT3 activation (17).

In the present studies we sought to identify the amino acid residues within IC3 that are critical for CRH-R1 signaling. We therefore introduced tandem and single alanine mutations by PCR-mediated mutagenesis, replacing amino acid cassettes at the juxtamembrane regions of IC3 or specific residues including basic amino acids (Arg^{292}, Lys^{297}, Arg^{299}, Arg^{310}, Lys^{311}, and Lys^{314}), and assessed the ligand binding and signaling characteristics of modified receptors. Our results demonstrate that both N- and C-terminal regions of the IC3 of CRH-R1 as well as basic amino acids possess specific structural elements with distinct contributions in G protein coupling and downstream cAMP production and ERK1/2 activation.

EXPERIMENTAL PROCEDURES

Reagents—CRH and Ucn1 were purchased from Bachem UK Ltd. (Helens, Merseyside, UK). Radiodinated Tyr^0-sauvagine was obtained from Amersham Biosciences. Dominant negative $Gα_I$ (Q205L/D273N) and $Gα_q$ (Q209L/D227N) were obtained from UMR cDNA Resource Centre, University of Missouri, Rolla. Forskolin was from Calbiochem/Merck. CRH-R1/2 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Phospho-ERK1/2 (Thr^{202}/Tyr^{204}) and total ERK1/2 were from Cell Signaling (Chandlers Ford, Hampshire, UK). The polyclonal G protein rabbit antibody AS/7 (anti-G_{i1}, G_{i2}), was obtained from NEN Life Science Products. Protein A-Sepharose beads (CL-4B) were purchased from Pharmacia Biotech. Secondary antibodies Alexa Fluor 405, Alexa Fluor 488, Alexa Fluor 594, and Alexa Fluor 680 were purchased from Invitrogen, and IRDye 800-conjugated goat anti-rabbit IgG was from Rockland Immunochemicals (Gilbertsville, PA). Cell culture media, Lipofectamine 2000, pcDNA3.1(−), restriction enzymes, and *Pfu* polymerase were from Invitrogen. dNTPs and DNA ladder were purchased from Bioline Ltd. (London, UK). Primers were purchased from TANG (Gateshead, UK). All other chemicals were purchased from Sigma-Aldrich.

Transfection of CRH-R1 to HEK293 Cells—Lipofectamine 2000 reagent was used for transfection of HEK293. Transient transfections were performed as described previously (18) when cells seeded in 25-cm^2 vented flasks reached 50–70% confluence. Approximately 24 h after transfection, cells were transferred to poly-D-lysine (Sigma)-coated glass coverslips (for confocal microscopy studies) or to 12-well plates for signaling studies. The experiments were performed 48 h after transfection. Receptor expression was verified by confocal microscopy analysis.

CRH-R1 Mutagenesis—A series of CRH-R1α mutants were generated by overlap extension PCR using high fidelity *Pfu* polymerase and mutagenic primers (supplemental Table 1) as described previously (20). The mutagenic CRH-R1 cDNA was cut sequentially with EcoRI and HindIII and then ligated back into the original pcDNA3.1(−). All mutant cDNAs were confirmed by sequence analysis of both strands (Molecular Biology Service, Department of Biological Sciences, University of Warwick).

CRH-R1 Radioreceptor Assay—Plasma membrane-rich fractions were prepared from HEK293 cells transiently expressing wild-type or mutant CRH-R1 as described previously (19). Radioiodinated Tyr^0-sauvagine (50 pM to 5 nM) binding to membranes (100 mg) was carried out for 2 h at room temperature, as described previously (19). Unlabeled oCRH (1000 molar excess) was used for displacement of radiolabeled Tyr^0-sauvagine. Nonspecific binding was 24 ± 5% of the total added radioactivity. The binding data were analyzed using the EBDA program (20) and LIGAND (21) (EBDA/LIGAND; Elsevier-Biosoft, Cambridge, UK).

Signaling Assays—ERK1/2 activation in response to 100 nM Ucn1 was determined in transiently transfected HEK293, seeded in 12-well plates to reach 60–70% confluence, as described previously using the Odyssey Infrared Imaging System (LI-COR Biosciences, Cambridge, UK), (19). Cyclic AMP production was measured as described previously (19) by a commercially available ELISA Direct Cyclic AMP Enzyme Immunoassay kit (Assay Designs Inc., Ann Arbor, MI). In some experiments Ucn1-induced cAMP production was determined following pretreatment of cells with either pertussis toxin (PTX) for 12 h, (final concentration, 100 ng/ml) or 20 mM mastoparan for 1 h, or the suramin analog NF023 (250 nM for 2 h).

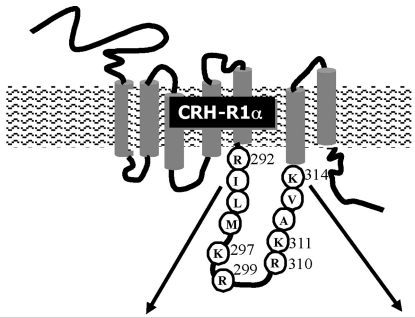
In some experiments, AC activity and inositol trisphosphate (IP_3) production were assessed using plasma membranes of HEK293 cells expressing wild-type or mutant receptors as

described previously (22). Briefly, cell membrane preparations (25 μg of protein) were incubated in triplicate with Ucn1 (100 nM) at 22 °C in the presence of 100 μl of 50 mM Tris-HCl buffer containing 10 mM $MgCl_2$, 1 mM EGTA, 1 g/liter BSA, 1 mM ATP, ATP regeneration system (7.4 mg/ml creatine phosphate, 1 mg/ml creatine phosphokinase), 100 μM 3-isobutyl-1-methylxanthine, pH 7.4. The reaction was terminated after 15 min by the addition of 1 ml of 0.1 M imidazole buffer, pH 7, followed by heating at 95 °C for 5 min. Cyclic AMP was measured by ELISA. For the IP_3 assay, cell membrane suspensions (40 μg) were incubated with increasing concentrations of Ucn1 followed by the addition of 200 μl of IP_3 generation buffer containing 25 mM Tris actetate buffer, pH 7.2, 5 mM Mg acetate, 1 mM DTT, 0.5 mM ATP, 0.1 mM $CaCl_2$, 0.1 mg/ml BSA, and 10 μM GTP. Membranes were incubated for 3 min at 37 °C, and the reaction was terminated by the addition of 1 M ice-cold trichloroacetic acid, followed by extraction of inositol phosphates and neutralization. IP_3 levels were estimated by a radioreceptor assay (PerkinElmer Life Sciences) based on the displacement of [^{3}H]IP_3 from calf cerebellum-derived membranes containing the IP_3-binding protein.

Plasma Membrane Fractionation and Co-immunoprecipitation Studies—For plasma membrane fractionation, cells were grown in 10-cm Petri dishes, and when 80% confluence was reached, cells were deprived of FCS for 1 h before treatment with 100 nM Ucn1 for 5 min. Membrane protein extracts were prepared using a ProteoExtract Native Membrane Protein Extraction kit (Calbiochem/Merck Biosciences). Protein concentration was determined using a BCA Protein Assay kit (Pierce). Protein fractions (5 μg) were analyzed by Western blot analysis using CRH-R1/2 antibodies (Santa Cruz Biotechnology) (dilution 1:100). Antibody binding was detected using horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (Dako) and ECL reagents from Amersham Biosciences. The relative density of the bands was measured by optical density scanning using the software Scion Image-Beta 3b for Windows (Scion Corp., Frederick, MD).

For co-immunoprecipitation studies, plasma membrane fractions were incubated overnight at 4 °C with antibodies specific for CRH-R1/2 (Santa Cruz Biotechnology) to a final concentration of 1:100 and protein A/G-agarose. The immunoprecipitates were washed four times in PBS, and proteins were eluted from the agarose beads by heating at 95 °C in Laemmli sample buffer. The resuspended proteins were analyzed by electrophoresis through 10% SDS-polyacrylamide gels and then electrophoretically transferred to PVDF membranes. Following blocking with 5% nonfat dry milk at room temperature for 30 min, membranes were subjected to immunoblot analysis by incubation at 4 °C for 2 h with $G\alpha_{i1/2}$ protein-specific antisera (dilution 1:200). After incubation with the secondary HRP-conjugated anti-rabbit IgG, proteins were visualized with ECL.

Data Analysis and Statistics—The relative involvement of $G\alpha_s$ and $G\alpha_i$ proteins on AC activation in HEK293 cells, transfected with wild-type or mutant CRH-R1α, was quantified as follows: cAMP responses (measured as Ucn1-induced cAMP -fold increase above basal) in control cells (R_T) were considered as responses involving both $G\alpha_s$ + $G\alpha_i$; cAMP responses in cells pretreated with PTX that inactivated $G\alpha_i$ proteins were considered as $G\alpha_s$-exclusive responses (R_s). The $G\alpha_i$ protein contribution (R_i) was calculated by using the equation: $R_i = R_s - R_T$. The results obtained are presented as the mean ± S.E. of each measurement. Data were tested for homogeneity, and comparison between group means was performed by one- or two-way ANOVA. Probability values of $p < 0.05$ were considered significant.



w.t CRH-R1α	----R^{292}-I-L-M^{295}---K^{297}---R^{299}	---R^{310}-K^{311}-A-V-K^{314}--
CRH-R1α (N-4A)	----A^{292}-A-A-A^{295}--K^{297}---R^{299}	---R^{310}-K^{311}-A-V-K^{314}--
CRH-R1α (C-4A)	----R^{292}-I-K-M^{295}---K^{297}---R^{299}	---R^{310}-A^{311}-A-A-A^{314}--
CRH-R1α (N-4A/C-4A)	----R^{292}-I-K-M^{295}---K^{297}---R^{299}	---R^{310}-A^{311}-A-A-A^{314}--
CRH-R1α (R292A)	----A^{292}-I-K-M^{295}---K^{297}---R^{299}	---R^{310}-K^{311}-A-V-K^{314}--
CRH-R1α (I293A)	----R^{292}-A-K-M^{295}---K^{297}---R^{299}	---R^{310}-K^{311}-A-V-K^{314}--
CRH-R1α (L294A)	----R^{292}-I-A-M^{295}---K^{297}---R^{299}	---R^{310}-K^{311}-A-V-K^{314}--
CRH-R1α (M295A)	----R^{292}-I-K-A^{295}----K^{297}---R^{299}	---R^{310}-K^{311}-A-V-K^{314}--
CRH-R1α (K297A)	----R^{292}-I-K-M^{295}---A^{297}---R^{299}	---R^{310}-K^{311}-A-V-K^{314}--
CRH-R1α (R299A)	----R^{292}-I-K-A^{295}----K^{297}---A^{299}	---R^{310}-K^{311}-A-V-K^{314}--
CRH-R1α (R310A)	----R^{292}-I-K-M^{295}---K^{297}---R^{299}	---A^{310}-K^{311}-A-V-K^{314}--
CRH-R1α (K311A)	----R^{292}-I-L-M^{295}---K^{297}---R^{299}	---R^{310}-A^{311}-A-V-K^{314}--
CRH-R1α (K314A)	----R^{292}-I-L-M^{295}---K^{297}---R^{299}	---R^{310}-K^{311}-A-V-A^{314}--

FIGURE 1. **Amino acid modifications in CRH-R1α mutant receptors.** Mutant receptors were generated with single or tandem amino acid Ala substitutions within the predicted IC3 amino acid sequence. The mutants were generated by overlap extension-PCR as described under "Experimental Procedures." An alanine residue was preferred due to lack of amino acid side chain beyond the carbon and thus able to determine the importance of specific amino acid side chains for receptor-G protein interactions. Furthermore, Ala substitution avoids potential problems associated with steric hindrance or unwanted ionic interactions, when amino acid residues with bulky side chains or charged groups are introduced.

RESULTS

Binding Properties of Mutant CRH-R1α Receptors—To identify residue(s) in the CRH-R1α N- and C-terminal regions of IC3 involved in receptor signaling, we used a tandem alanine scanning mutagenesis strategy, in which four consecutive amino acid residues were replaced by alanines (Fig. 1). A series of additional mutants was also generated containing single Ala substitutions of residues in the proximal (Arg^{292}-Met^{295}) and distal (Lys^{311}-Lys^{314}) terminal segments, as well as the charged amino acids Lys^{297}, Arg^{299}, Arg^{310}. Mutant and wild-type receptors were transiently expressed in HEK293 cells, and immunoblotting experiments using a specific CRH-R1/2 Ab demonstrated that levels of expression at the cell membrane were similar (supplemental Fig. 1). Most mutants retained high binding affinity for the radiolabeled agonist ^{125}I-Tyr^0-sauvagine (supplemental Table 2), comparable with the wild-type CRH-R1α (data not shown), indicating retention of the normal conformation of the wild-type receptor. However, mutant CRH-R1α(N-4A/C-4A), where both N- and C-juxtamembrane regions of IC3 were substituted to Ala, showed a 5–8-fold

increase in IC_{50} compared with the wild-type receptor (8.79 ± 2.93 *versus* 1.26 ± 0.76 nM, respectively), indicating reduced ligand binding affinity. Cell membranes expressing wild-type or mutant CRH-R1α receptors exhibited comparable levels of total amount of binding sites (B_{max}) available for agonist binding (average 865 ± 60 ranging from 714 ± 36 to 980 ± 135 fmol/mg of protein, respectively, as determined by Scatchard analysis of ^{125}I-Tyr^0-sauvagine binding to plasma membranes) (supplemental Table 2). By contrast, the B_{max} of overexpressed CRH-R1α(N-4A/C-4A) mutant was also found to be decreased by 15–20% ($p < 0.05$).

Structural Motifs within IC3 of CRH-R1α IC3 Involved in Activation of AC—Our previous studies (26) demonstrated that although the CRH-R1α can activate multiple classes of G proteins, it is most efficiently coupled to Gα_s proteins leading to downstream activation of AC and elevation of intracellular cAMP levels (24). The role of the juxtamembrane regions of the IC3 predicted amino acid sequence in Ucn1-induced cAMP production was further investigated in HEK293 cells transiently expressing wild-type or mutant CRH-R1α. Introduction of tandem Ala mutations in either the Arg^{292}-Met^{295} or Lys^{311}-Lys^{314} terminal regions of the IC3 (mutants CRH-R1α(N-4A) and CRH-R1α(C-4A), respectively) significantly increased maximal cAMP production stimulated by Ucn1, by 78 and 87% respectively, compared with the wild-type receptor (Fig. 2*a*). These mutations did not substantially alter the EC_{50} values of mutant receptors (supplemental Table 3). However, mutant CRH-R1α(N-4A/C-4A) demonstrated a 90% decrease in cAMP response compared with the wild-type receptor, suggesting that disruption of IC3 secondary structure severely impaired receptor ability to couple to G proteins crucial for AC activation. Interestingly, the N- and C-tandem Ala mutations altered the shape of agonist dose-response curves; although the wild-type receptor exhibited a biphasic pattern of responses where maximum cAMP production at 100 nM Ucn1 was followed by a slightly reduced response at higher agonist concentration (1 mM), mutant receptor responses were characterized by a plateau at the maximum response with no apparent reduction observed at higher Ucn1 concentrations. These changes might reflect alterations (reduction) in inhibitory G protein contribution to AC activation in the mutants compared with the wild-type receptor at high Ucn1 concentrations.

To identify specific amino acid residues important for G protein coupling and AC activation, we examined agonist-induced cAMP responses in a series of mutant CRH-R1α receptors that contained single Ala substitutions throughout the N- and C-terminal segments of IC3 as well as other charged amino acids. We found that single Ala substitution of any of the five charged amino acids (Arg^{292}, Lys^{297}, Arg^{310}, Lys^{311}, or Lys^{314}) significantly increased Ucn1-induced maximal cAMP production, without affecting EC_{50} values of cAMP responses (EC_{50} (nM) = 4 ± 0.8) (Fig. 2*b*). Receptor activation of cAMP was increased by 70% over wild-type when Arg^{292} or Lys^{311} was replaced by alanine whereas Lys^{297}, Arg^{310}, or Lys^{314} substitution led only to a 50% increase in cAMP production. A significant increase in cAMP response, albeit to a lesser degree, was also observed when either Lys^{294} or Met^{295} was replaced by alanine (25 and 30% increase of wild-type receptor cAMP response, respectively). In contrast, Ala substitution of Arg^{299} reduced by 20–25% maximum cAMP production, again without affecting EC_{50} value of cAMP response.

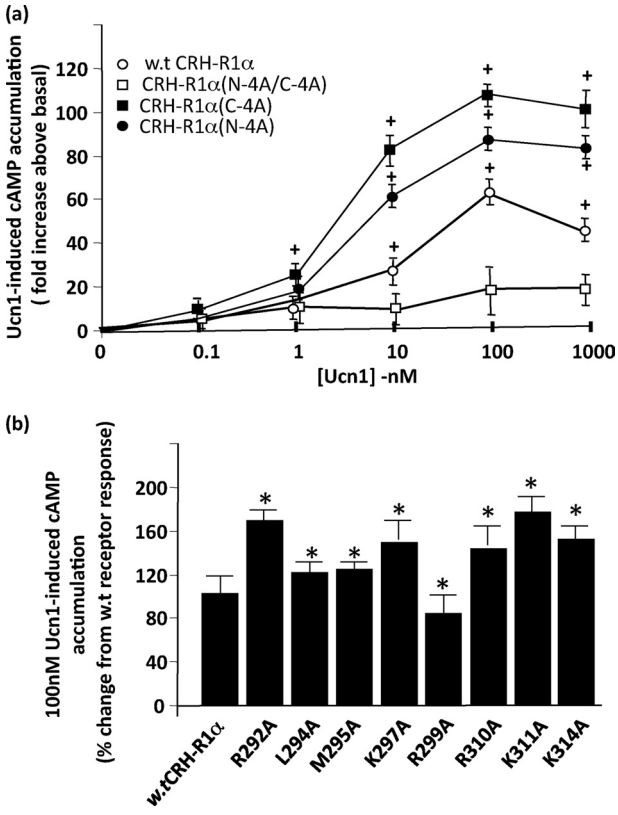


FIGURE 2. **Cyclic AMP signaling characteristics of wild-type and mutant CRH-R1α receptors.** HEK293 cells, transiently transfected with wild-type or mutant CRH-R1α as described under "Experimental Procedures," were stimulated with different concentrations of Ucn1 (0.1–1000 nM) (*a*) or 100 nM Ucn1 for 15 min to determine dose-dependent characteristics (EC_{50}) and maximal responses (*b*). Ucn1-induced cAMP accumulation was determined by ELISA (measured as Ucn1-induced cAMP -fold increase above basal). Data represent the mean ± S.E. (*error bars*) of three estimations from three independent experiments. +, $p < 0.05$ compared with basal (unstimulated); *, $p < 0.05$ compared with maximal wild-type response. Results were normalized to maximum forskolin response.

Previous studies (4) suggested that the CRH-R1 can adopt distinct, agonist-specific, active conformations that result in important differences in G protein interaction and downstream signal effects. Hence, we used CRH and Ucn1 to activate CRH-R1α mutants to investigate whether Ala substitutions resulted in agonist-specific differences on receptor ability to stimulate AC activity. For all mutants tested (R292A, L294A, M295A, R299A, K311A, and K314A), no significant differences were found between CRH and Ucn1 maximal (Fig. 3) or EC_{50} (supplemental Table 3) values of cAMP responses, suggesting a similar role for these amino acid residues in the receptor active conformation, induced by CRH and Ucn1, required to activate the pathway leading to increased intracellular cAMP levels. Similar agonist responses were observed when mutants I293A, K297A, and R310A were transiently expressed in HEK293 cells (data not shown).

In addition to high affinity coupling to Gα_s, CRH-R1α receptors can also couple to Gα_i proteins especially at nanomolar agonist concentrations (9, 26). Hence, it is likely that the net activation of AC reflects the opposing actions of these G pro-

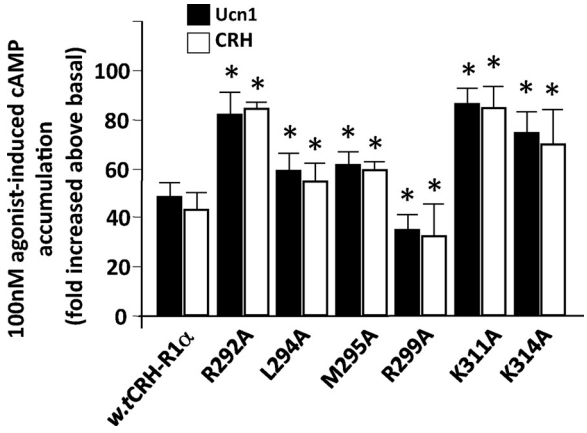


FIGURE 3. **Comparison of CRH-R1α receptor agonists potency to activate AC in HEK293 cells expressing wild-type and mutant CRH-R1α receptors.** Cells were stimulated with either 100 nM Ucn1 or CRH for 15 min to induce maximal AC activation, and cAMP accumulation was measured by ELISA. Data represent the mean ± S.E. (*error bars*) of three estimations from three independent experiments. *, $p < 0.05$ compared with maximal wild-type response. Results were normalized to maximum forskolin response.

teins. Indeed, our preliminary experiments (supplemental Fig. 2*a*) demonstrated that at agonist concentrations greater than 1 nM, PTX pretreatment enhanced Ucn1-induced AC activation, without any change in the bell-shaped dose-response curve; this suggests a role for $G_{i/o}$-mediated inhibition of AC activation in addition to G_s-dependent stimulation, especially at high agonist concentrations that correspond to the G_i-coupled phase, as described previously (9). By contrast, at subnanomolar concentrations Ucn1 effects were exclusively mediated via G_s-induced AC activation, a result consistent with the lower coupling affinity of the receptor to G_i proteins (25). The involvement of $G_{i/o}$ proteins in receptor-mediated cAMP responses was also investigated in experiments employing mastoparan, the wasp venom-derived peptide, which can selectively activate $G_{i/o}$ proteins (27). Mastoparan pretreatment attenuated Ucn1-induced cAMP responses in control cells (supplemental Fig. 2*a*), consistent with receptor-independent activation of $G_{i/o}$ proteins and inhibitory actions on Ucn1-induced AC activation. However, mastoparan had no effect in cells expressing a Gα_i dominant negative (DN) (Q205L/D273N) (supplemental Fig. 2*b*), thus providing further evidence that $G_{i/o}$ proteins are involved in mastoparan regulation of AC activity. Ucn1-induced cAMP responses were also sensitive to NF023, a selective $G_{i/o}$ protein inhibitor, and cells pretreated with 250 nM NF023 exhibited increased agonist-stimulated cAMP responses (supplemental Fig. 2*c*). Interestingly, the bell-shaped response remained detectable even after treatment with either PTX or mastoparan, suggesting that the inhibitory phase was partially independent of receptor coupling to G_i.

These results led us to examine the possibility that the enhanced cAMP responses observed in mutants R292A, K297A, R310A, K311A, and K314A or the reduced cAMP response of R299A was due to altered coupling of receptors to Gα_s and/or Gα_i proteins. Cells expressing wild-type or mutant receptors were pretreated with or without PTX (to inactivate $G_{i/o}$ proteins) prior to agonist stimulation and AC activation. The effectiveness of PTX in inactivating $G_{i/o}$ proteins has been previously confirmed by demonstrating inhibition of agonist-induced labeling of Gα_i proteins with the nonhydrolyzable GTP analog [α-^{32}P]GTP-AA (25). Results (Fig. 4*aI*) showed that wild-type CRH-R1α activation by 100 nM Ucn1, to induce maximal cAMP production, involved Gα_s proteins as well as PTX-sensitive G proteins; the latter exerted an inhibitory effect (by 18% Ri component) on AC activity, as demonstrated by an increased agonist-induced (but not basal) cAMP response in PTX-treated cells that unmasked the full activity of Gα_s proteins. We used the same experimental paradigm in cells expressing receptor mutants, which exhibited altered cAMP responses (Fig. 2). In K314A-expressing cells (Fig. 4*a*), we found that PTX pretreatment enhanced Ucn1-induced cAMP production by 20% above control (untreated) cells, indicating similar to the wild-type, R_i responses. This suggests that the alanine substitution at position Lys^{314} had negligible effects on receptor Gα_i protein coupling and that the increased R_s response resulted from a more efficient coupling to Gα_s proteins. By contrast, in CRH-R1α(R292A), CRH-R1α(K297A), CRH-R1α(R310A), and CRH-R1α(K311A), uncoupling of Gα_i protein activation by PTX pretreatment did not significantly modify cAMP production in response to 100 nM Ucn1 (Fig. 4*b*), suggesting that these mutant receptors exhibited minimal Gα_i protein coupling. Similarly, CRH-R1α(R299A) response was not affected by PTX pretreatment, suggesting negligible Gα_i protein coupling and contribution to the overall cAMP response (Fig. 4*aII*). Thus, it appears that this mutation can attenuate Gα_i protein coupling as well as reduce receptor R_s response, suggesting that the Arg^{299} residue is required for optimal interaction with Gα_s proteins.

To confirm the increased Gα_s protein coupling in mutant receptors R292A, R310A, K311A, and K314A, we stimulated wild-type or mutant receptors with an Ucn1 concentration (0.5 nM) lower than the concentration threshold required to activate Gα_i proteins (1–10 nM). All mutants exhibited increased agonist-induced cAMP production compared with wild-type receptor responses (Fig. 4*b*) that were not altered by PTX pretreatment, confirming absence of any significant contribution from R_i responses and suggesting involvement primarily of Gα_s proteins ($R_T = R_s$ response).

We next considered the possibility that altered receptor-G protein coupling efficiency and AC activation in the mutant receptors was due to modification of intracellular mechanisms downstream of receptor-G protein interactions. This was evaluated by assessing Ucn1-induced activation of AC (with or without PTX pretreatment) in cell membranes isolated from HEK293 cells transiently expressing wild-type CRH-R1α or mutant receptors. The results of these studies and studies of cAMP accumulation were similar (supplemental Fig. 3), confirming that the mutation-related effects on receptor signaling characteristics involved events near or at the plasma membrane that altered receptor-G protein interactions.

Structural Motifs within IC3 of CRH-R1α Involved in Activation of ERK1/2—In addition to AC, CRH-R1α can also activate the ERK1/2 signaling cascade (26, 28). A number of different G proteins appear to be involved in CRH-R1α-induced activation of ERK1/2; for example Gα_i- and Gα_q-mediated pathways stimulate, whereas Gα_s/AC and protein kinase A (PKA) limit maximal ERK1/2 phosphorylation (5). Hence, we assessed the

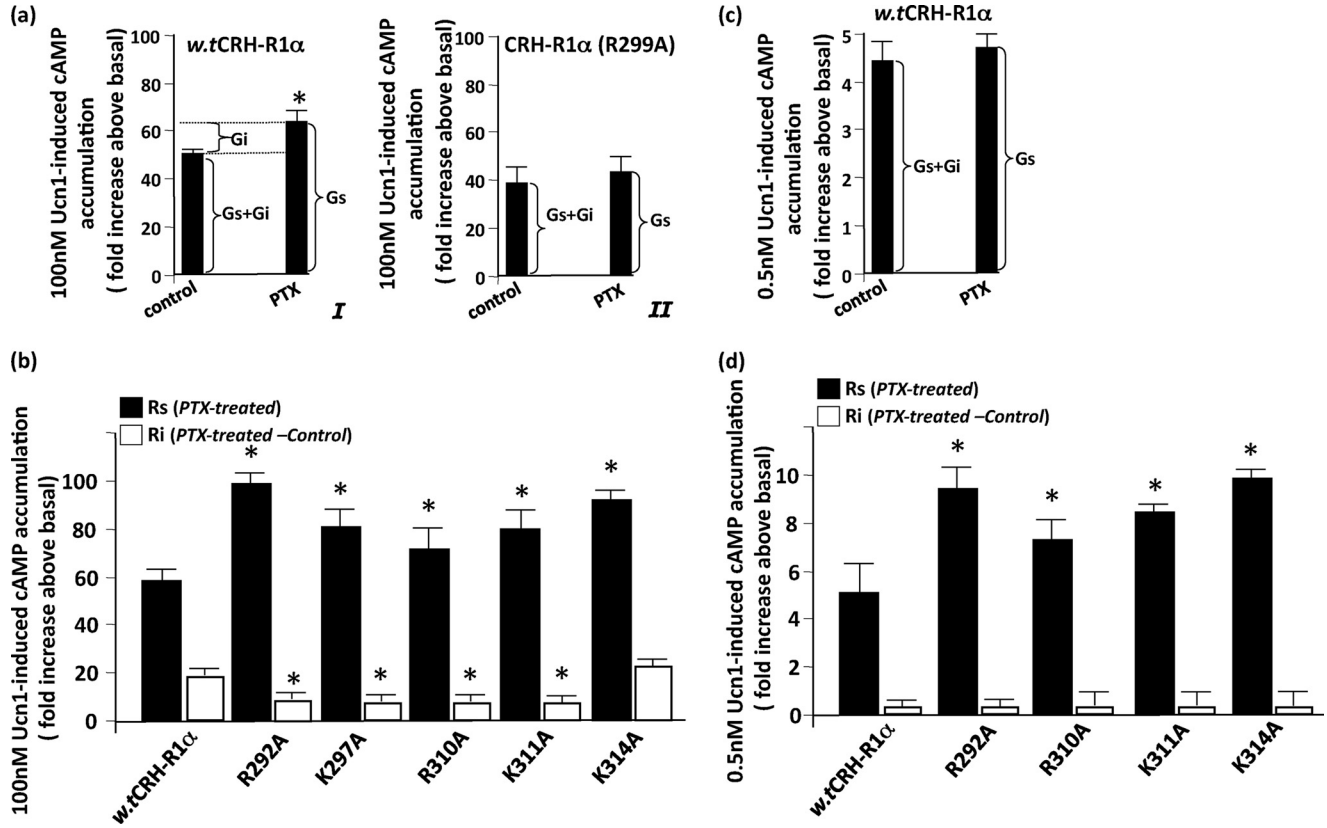


FIGURE 4. **Effect of PTX treatment on Ucn1-induced AC activation in HEK293 cells expressing wild-type and mutant CRH-R1α receptors.** Cells were pretreated with PTX for 12 h (final concentration, 100 ng/ml) to inactivate $G_{i/o}$ proteins. In cells expressing wild-type receptors (control cells; *al*), Ucn1-induced cAMP responses (measured as Ucn1-induced cAMP -fold increase above basal) were considered as total responses (R_T) involving both $G_s + G_i$; cAMP responses in cells pretreated with PTX were considered as G_s-only responses (R_s). The G_i protein contribution (R_i) was calculated by using the equation: $R_i = R_s - R_T$. The R_s and R_i parameters were determined for a number of mutant CRH-R1α receptors stimulated with either 100 nM (*a*) or 0.5 nM (*b*) Ucn1. *al*, wild-type receptor cAMP responses; *all*, CRH-R1α(R299A) receptor cAMP responses. *b*, cAMP responses induced by 100 nM Ucn1 following PTX pretreatment in wild-type and mutant CRH-R1α receptors. *c* and *d*, cAMP responses induced by 0.5 nM Ucn1 following PTX pretreatment of cells expressing wild-type (*c*) or mutant CRH-R1α receptors (*d*). Data represent the mean ± S.E. (*error bars*) of three estimations from three independent experiments. *, $p < 0.05$ compared with maximal wild-type response. Results were normalized to maximum forskolin response.

potential involvement of amino acid residues within receptor IC3 in pathways leading to ERK1/2 phosphorylation. Cells expressing CRH-R1α(N-4A) or CRH-R1α(C-4A) receptors had significantly reduced maximal ERK1/2 phosphorylation in response to Ucn1(100 nM) treatment (reduced by 70 and 77%, respectively, maximal stimulation 3 ± 0.5 and 2.8 ± 0.3-fold above basal), compared with the wild-type receptor (Fig. 5*a*). Significant ERK1/2 phosphorylation was observed only at high (100 nM) agonist concentrations, however, suggesting impaired receptor sensitivity in activating this pathway. Similar to cAMP results described above, mutant CRH-R1α(N-4A/C-4A) signaling activity was markedly impaired and showed no measurable ERK1/2 phosphorylation upon agonist stimulation, again highlighting the impact of the mutations on IC3 function.

We also assessed the importance of specific amino acids within IC3 in determining the ability of CRH-R1α to activate ERK1/2. By measuring Ucn1-induced stimulation of ERK1/2 phosphorylation in HEK293 cells expressing mutant receptors, we found that all mutant receptors had variably reduced ability to transduce agonist activation of ERK1/2. The I293A substitution resulted in the most dramatic effect, as ERK1/2 activation was diminished by 70%; all other mutant receptors exhibited loss of activity by 30–60% (Fig. 5*b*), indicating that these amino acid residues are each important for receptor coupling to the ERK1/2 pathway. It is noteworthy that the CRH-R1α(C-4A) mutant, which is essentially a K311A/K314A double mutant, was significantly more impaired in ERK1/2 responses than either the K311A or K314A mutant alone, suggesting an interaction between the two native amino acids. Because our previous observations suggested that specific amino acids within Arg^{292}-Lys^{297} and Arg^{310}-Lys^{314} terminal regions (especially residues Arg^{292}, Lys^{297}, Arg^{310}, and Lys^{311}) as well as Arg^{299}, were important for CRH-R1α coupling to PTX-sensitive G proteins, we next investigated the role of $G_{i/o}$ proteins in wild-type- and mutant receptor-mediated ERK1/2 activation. Pretreatment of cells expressing wild-type CRH-R1α with PTX resulted in a significant attenuation by 50–60% of maximal ERK1/2 phosphorylation induced by 100 nM Ucn1 (Fig. 6*a*). This result indicated substantial involvement of $G_{i/o}$ proteins in ERK1/2 activation, in agreement with previous studies (4, 25) as well as contribution of other pathways potentially involving PTX-insensitive G proteins and/or G protein-independent mechanisms, as demonstrated previously (26).

Altered coupling to PTX-sensitive G proteins might explain the reduced potency of mutant CRH-R1α receptors in activating ERK1/2 phosphorylation. With the exception of mutants CRH-R1α(R292A), CRH-R1α(K297A), CRH-R1α(R310A), and CRH-R1α(K311A), the reduced ability of mutant receptors to

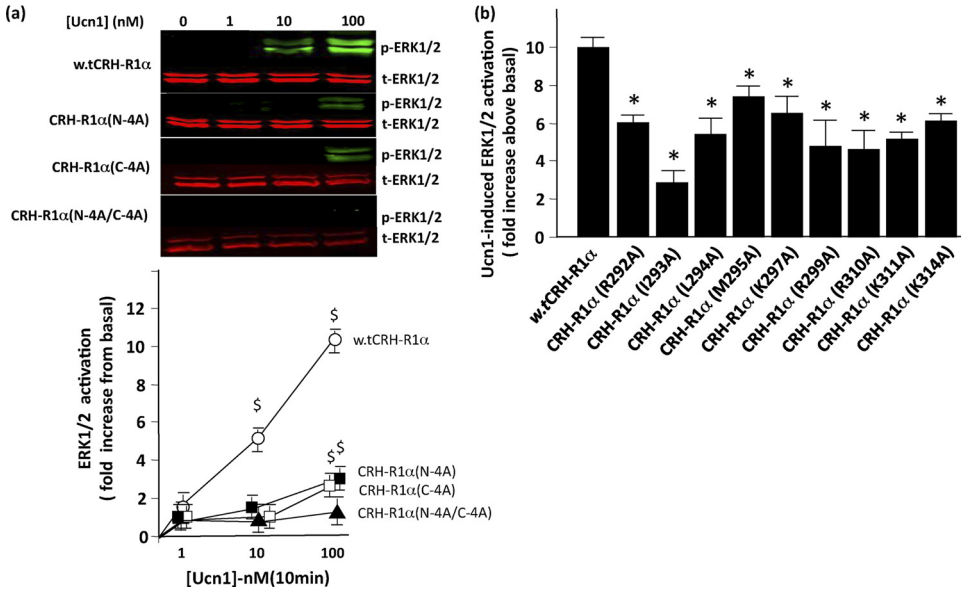


FIGURE 5. **ERK1/2 activation in HEK293 cells expressing wild-type and mutant CRH-R1α receptors.** HEK293 cells, transiently transfected with wild-type or mutant CRH-R1α as described under "Experimental Procedures," were stimulated with different concentrations of Ucn1 (0.1–1000 nM) (*a*) or 100 nM Ucn1 for 5 min to determine dose-dependent characteristics and maximal responses of ERK1/2 activation (*b*). Following cell lysis and centrifugation, supernatants were subjected to SDS-PAGE and immunoblotted with primary antibodies for phospho-ERK1/2 (Thr^{204}/Tyr^{202}) and total ERK2 to determine the phosphorylated/activated ERK1/2 and secondary antibodies conjugated to IRDye 800 and Alexa Fluor 680 (near infrared fluorophore dyes). *Top panels* in *a* are representative Western blots of Ucn1-induced ERK1/2 activation in cells expressing wild-type CRH-R1α receptor. Data represent the mean ± S.E. (*error bars*) of three estimations from three independent experiments. \$, $p < 0.05$ compared with basal; *, $p < 0.05$ compared with wild-type response.

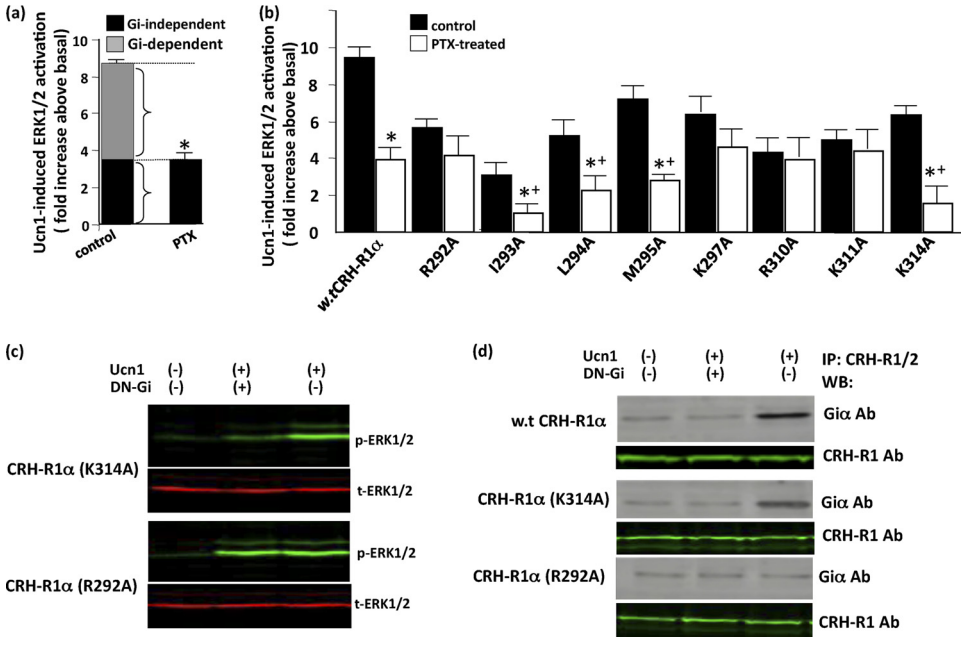


FIGURE 6. **Role of G_i proteins in Ucn1-induced ERK1/2 activation in HEK293 cells expressing wild-type and mutant CRH-R1α receptors.** *a* and *b*, cells expressing wild-type or mutant receptors were pretreated with or without PTX for 12 h (final concentration, 100 ng/ml) to inactivate $G_{i/o}$ proteins. In cells pretreated with PTX, ERK1/2 responses were considered as G_i-independent-only responses whereas ERK1/2 responses in control cells were considered as total (G_i-dependent and G_i-independent) responses. Data represent the mean ± S.E. (*error bars*) of three estimations from three independent experiments.. +, $p < 0.05$ compared with wild-type PTX-treated response; *, $p < 0.05$ compared with control response. *c* and *d*, in some experiments, Ucn1-induced ERK1/2 activation was determined in cells co-expressing mutant receptors and DN $G\alpha_i$ (Q205L/D273N). Furthermore, membrane-rich fractions from cells expressing wild-type or mutant receptors were prepared using a ProteoExtract Native Membrane Protein Extraction kit as described under "Experimental Procedures," solubilized, and immunoprecipitated with specific CRH-R1/2 antibodies. Proteins were resolved on SDS-polyacrylamide gels followed by immunoblotting with $G\alpha_{i1/2}$ antibodies to identify potential complex formation. CRH-R1 immunoblotting was also carried out as loading control. *Panels* in *c* and *d* are representative Western blots. Identical results were obtained from three independent transfection experiments.

activate the ERK1/2 pathway was further attenuated by PTX pretreatment (Fig. 6*b*). The CRH-R1α(R299A) response was also resistant to PTX pretreatment (data not shown). These results indicated that mutant receptors could be broadly subdivided into two groups: (i) mutants CRH-R1α(R292A), CRH-R1α(K297A), CRH-R1α(R299A), CRH-R1α(R310A), and CRH-R1α(K311A), with little or no coupling to PTX-sensitive G proteins (*i.e.* with minimal contribution to ERK1/2 phospho-

rylation by G_i proteins; and (ii) mutants CRH-R1α(I293A), CRH-R1α(L294A), CRH-R1α(M295A), and CRH-R1α-(K314A), with substantial coupling to PTX-sensitive G_i proteins. In particular, mutants CRH-R1α(I293A) and CRH-R1α(K314A) exhibited negligible ERK1/2 phosphorylation activity when G_i proteins were uncoupled by PTX, suggesting minimal involvement by non-G_i protein-dependent pathways.

We used two complementary approaches to confirm that mutant receptors had altered coupling to G proteins after Ucn1 activation: overexpression of a DN $G\alpha_i$ (Q205L/D273N) in HEK293 cells and co-immunoprecipitation studies to demonstrate altered receptor G_i protein coupling following Ucn1 activation. We found that Ucn1 stimulation of HEK293 cells expressing CRH-R1α(K314A) and DN $G\alpha_i$ protein (Q205L/D273N) had significantly reduced (by 70–80%) ERK1/2 phosphorylation, which further supported the involvement of $G\alpha_i$ protein in receptor-mediated response. By contrast, the DN $G\alpha_i$ protein had no effect on CRH-R1α(R292A)-mediated ERK1/2 phosphorylation (Fig. 6*c*). Similarly, after Ucn1 (100 nM) treatment, there was increased co-immunoprecipitation of $G\alpha_{i1/2}$ proteins with wild-type or CRH-R1α(K314A) receptor proteins (Fig. 6*d*). Furthermore, when the DN $G\alpha_i$ protein was co-expressed in HEK293 cells, Ucn1 treatment did not increase the amount of $G\alpha_i$ protein precipitated. In agreement with our previous observations in membranes from cells expressing CRH-R1α(R292A), the agonist had a minimal effect on receptor-G_i protein association, and only small amounts of $G\alpha_i$ protein were co-precipitated with the receptor.

CRH-R1α IC3 Residues Important for Phospholipase C Activation and IP_3 Production—CRH-R1α-induced activation of ERK1/2 also involves PTX-insensitive $G\alpha_q$-mediated pathways that lead to downstream activation of phospholipase C and intracellular IP_3 accumulation (4). Therefore, we assessed the potential involvement of amino acid residues within receptor IC3 in pathways leading to IP_3 production. In agreement with previous studies (4), treatment of cell membrane suspensions isolated from cells expressing wild-type CRH-R1α with Ucn1 (1–250 nM), stimulated a significant increase in IP_3 production by 220–250%, with a threshold of 10 nM and a maximum response at 100 nM (data not shown). Previous studies (9) suggested that CRH-R1α-induced IP_3 accumulation involves $G\alpha_q$-mediated pathways with contribution from PTX-sensitive pathways via $G\beta\gamma$ dimers released from heterotrimeric G_i proteins. Our results supported this because PTX treatment caused a small but significant reduction by 15–20% in Ucn1-induced IP_3 production (Fig. 7*a*). Cell membranes prepared from HEK293 cells co-expressing CRH-R1α and DN $G\alpha_q$ (Q209L/D227N) had significantly reduced (by 89 ± 5%) IP_3 accumulation, thus confirming the involvement of $G\alpha_q$ proteins in receptor-mediated IP_3 response.

Investigation of the ability of mutant receptors to induce IP_3 production identified that mutation of residues Ile^{293} and Lys^{314} significantly impaired receptor sensitivity and maximal response, whereas mutation of residues Leu^{294}, Met^{295}, and Lys^{297} reduced maximal response without affecting receptor sensitivity (Table 1). Compared with the wild-type receptor, these mutant receptors exhibited reduced IP_3 production even after PTX pretreatment (Fig. 7*b*), thus confirming that these mutations mainly modified receptor coupling to PTX-insensitive G proteins involved in IP_3 production, most likely $G\alpha_q$ protein. All other mutant receptors showed IP_3production comparable with that of the wild-type receptor.

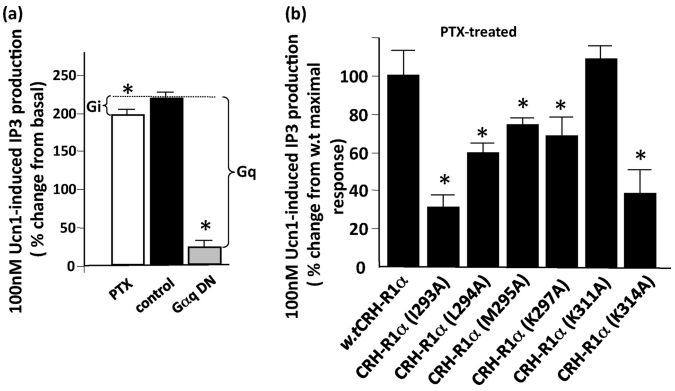


FIGURE 7. **IP_3 signaling characteristics of wild-type and mutant CRH-R1α receptors.** *a*, cell membrane suspensions (40 μg) were prepared from cells expressing wild-type receptors and co-expressing DN $G\alpha_q$ (Q209L/D227N) using a ProteoExtract Native Membrane Protein Extraction kit as described under "Experimental Procedures." Membranes were pretreated with or without (control) PTX for 12 h (final concentration, 100 ng/ml) to inactivate $G_{i/o}$ protein-dependent pathways. *b*, similar experiments were carried out in cells expressing mutant CRH-R1α receptors. Subsequently, membranes were stimulated with 100 nM Ucn1 for 30 min at room temperature, followed by the addition of 200 μl of IP_3 generation buffer and further incubation for 3 min at 37 °C. This was followed by extraction of inositol phosphates and neutralization. IP_3 levels were determined by competitive binding assay. Results are expressed as the mean ± S.E. (*error bars*) of three estimations from three independent experiments. *, $p < 0.05$ compared with untreated (control) membranes (*a*) or wild-type CRH-R1α (*b*).

TABLE 1

CRH-R1 agonist-induced IP_3 production in HEK293 cells expressing wild-type and alanine mutants of CRH-R1α

HEK293 cells transfected with wild-type or IC3 mutant CRH-R1α receptor cDNAs were assayed for Ucn1-stimulated IP_3 production 72 h after transfection as described under "Experimental Procedures." Cell membrane suspensions (40 μg) were prepared and treated with different concentrations of Ucn1 ($0–10^{-6}$ M) for 30 min at room temperature, followed by the addition of 200 μl of IP3 generation buffer and further incubation for 3 min at 37 °C. This was followed by extraction of inositol phosphates and neutralization. IP3 levels were determined by competitive binding assay. Results are expressed as the mean ± S.E. of four estimations from three independent experiments. *, $p < 0.05$ compared with wild-type CRH-R1α.

Receptor	IP_3	
	EC_{50}	Maximal response
	nM	*% of WT receptor*
Wild-type CRH-R1α	50 ± 5	100 ± 11
CRH-R1α (R292A)	55 ± 20	93 ± 16
CRH-R1α (I293A)	88 ± 18*	30 ± 13*
CRH-R1α (L294A)	58 ± 24	60 ± 6*
CRH-R1α (M295A)	45 ± 15	70 ± 7*
CRH-R1α (K297A)	60 ± 9	50 ± 20*
CRH-R1α (R299A)	40 ± 11	90 ± 17
CRH-R1α (R310A)	55 ± 25	97 ± 8
CRH-R1α (K311A)	60 ± 15	101 ± 21
CRH-R1α (K314A)	90 ± 27*	39 ± 13*

DISCUSSION

Despite significant advances in understanding the structural determinants that affect binding of CRH-R1 to its cognate agonists CRH and Ucn1, the structural motifs important for determining the ability of CRH-R1 to trigger distinct signaling responses remain poorly defined. Our study focused on the CRH-R1 proximal and distal terminal segments of IC3, because for many GPCRs, the cationic α-helices in the juxtamembrane regions near transmembrane domains (TMDs) 5 and 6 appear

to be major functional domains for receptor-G protein coupling and generation of downstream intracellular responses. In particular, the motifs *B-B-X-B*, or *B-X-X-B* where *B* is a basic residue and *X* is a nonbasic residue, have been identified as important for activation of G proteins and signal transduction as well as receptor maturation and expression (29–35). This motif, primarily detected in the IC3-TM6 junction, is one of the few structurally conserved elements with established functional role shared between the A and B families of GPCRs. Sequence alignment suggests that all B1 GPCRs family members contain the sequence K/R-A/L-*X*-K/R (Fig. 7*a*). Moreover, in 11 of 15 class B1 GPCRs, including the CRH-R1, the TM5-IC3 junction contains the conserved hydrophobic motif *B*-I/L/V-L-*X*, which also appears to be crucial for efficient G protein coupling.

Site-specific mutations within the terminal segments of IC3 of the hCRH-R1α were constructed and used to probe the role of these domains in coupling to G proteins and signaling pathways that activate the cAMP/PKA and ERK1/2 pathways. One striking finding was that simultaneous disruption of the cationic α-helices near TMD 5 and 6 by tandem Ala substitutions, and presumably alteration of the conformation and orientation of IC3, abolished the ability of CRH-R1α to transduce signals across the cell, highlighting the important role of IC3 for G protein coupling. The CRH-R1α(N-4A/C-4A) mutant also exhibited reduced agonist binding affinity which might be due to an alteration of the overall conformation of receptor associated with a disrupted G protein/receptor interaction. Agonist interaction with the G protein-uncoupled CRH-R1 has been shown to weakly stabilize agonist binding, whereas efficient G protein coupling was required for stabilization of agonist-receptor interaction and high affinity binding (36).

Our results also suggest that the cationic α-helices Arg^{292}-Lys^{297} and Arg^{310}-Lys^{314} contain specific amino acids that are important determinants of CRH-R1α coupling to specific G proteins and activation of the AC /cAMP pathway, a crucial signaling event for the activation of the HPA axis and the stress response. The role of these residues in regulating formation of the receptor-G protein ternary complex was evident only in the agonist-activated state, as mutants did not exhibit altered basal (constitutive) activity. Our study showed that within these microdomains, charged amino acids such as Arg^{292}, Lys^{297}, Arg^{310}, Lys^{311}, and Lys^{314}, as well as hydrophobic amino acids Leu^{294} or Met^{295} (albeit to a lesser degree) are likely key determinants of the primary receptor recognition sites for G_s and G_i protein as well as G_q protein coupling. Because amino acid substitutions (Leu/Met → Ala) of the latter group would abolish hydrophobicity without a significant effect on receptor conformation, this raises the possibility that the primary structure and aliphatic side chain of Leu^{294} and Met^{295} is important for receptor IC3-G protein interactions. As mentioned above, these cationic α-helical domains have been studied extensively in both family A and B GPCRs and in most cases are required for efficient G protein interaction and signal transduction. It has been proposed that for some GPCRs, the helix stabilizes the wall of the G protein pocket by packing against the distal portion of IC3 (37). Surprisingly, we uncovered an unexpected role of these amino acids because disruption of each of these terminal segments enhanced cAMP signaling, suggesting an alternative, not previously described, function of these amino acid clusters that restrain CRH-R1α ability to activate the AC/cAMP pathway. The position of charged amino acids within IC3 also appears to be important for determining their contribution to G protein coupling: charged amino acids such as Arg^{299} appear to promote efficient receptor IC3-G protein interactions. We showed previously (5) that the ability of the CRH-R1α receptor to activate this pathway is determined by the opposing actions of G_s and G_i proteins, thus the effects of the Ala mutations might be explained in the context of either removal of putative conformational constraints of the secondary structure that optimize the G_s protein binding pocket or, alternatively, disruption of the G_i protein binding pocket, or both. Indeed, our studies showed that specific amino acids such as Arg^{292}, Arg^{310}, and Lys^{311} are key functional determinants of IC3 interaction with both G_s and G_i proteins, whereas other amino acids in this region, such as Leu^{294}, Met^{295}, and Lys^{314} exhibit G_s protein interaction selectivity (Fig. 8*b*). Our previous studies (5) have shown that the position of charged amino acid residues within IC3 is critical for receptor G protein coupling: for example introduction of a basic charged amino acid at position 301 within the Ser/Thr amino acid cluster (Ser^{301}-Thr^{306}) of IC3 impairs the ability of CRH-R1α to couple efficiently to G_s proteins, whereas substitution of Ser^{301} with an acidic amino acid abolishes G_i protein activation.

This differential contribution of specific amino acids depending on the relative position within IC3 could be associated with the ability of the receptor to adopt different conformations. It has been proposed that the CRH-R1 can adopt at least two distinct configurations, which couple to G_s or G_i protein (40). Ala mutation at the Arg^{292} or Lys^{314} position might alter receptor-G_s protein interaction due to either loss of charge or a hydrophilic residue that helps anchor the receptor at the plasma membrane and thus might allow IC3 to a conformation that unmasks G_s protein interaction points or possibly favors interaction with other GPCR- interacting proteins. The importance of basic residues in receptor interactions with interacting protein has been established; for example an arginine cluster within IC3 of the vasopressin V_2 receptor has been identified as the motif important for receptor interaction with GC1q-R (41).

Recent crystallization of the β_2-adrenergic receptor-G_s complex provided the first high resolution insight into the mechanism of signal transduction across the plasma membrane by a GPCR (38, 39). These studies identified that movements of TMD5 and 6 that destabilize the receptor inactive state and result in conformational changes of IC3 are crucial steps for β_2-adrenergic receptor-G_s interaction. Therefore, it is possible that the CRH-R1 IC3 amino acids identified in this study play unique roles in this mechanism of complex formation and coupling to distinct G proteins.

An important determinant of CRH-R1α-G protein interactions that determine the bell-shaped cAMP response appears to be the activating concentration of the agonist. In our studies, subnanomolar Ucn1 concentrations failed to induce receptor-G_i protein interactions, and all IC3 mutations tested did not alter these characteristics. Interestingly, comparison of dose-response curves between mutant and wild-type receptors and

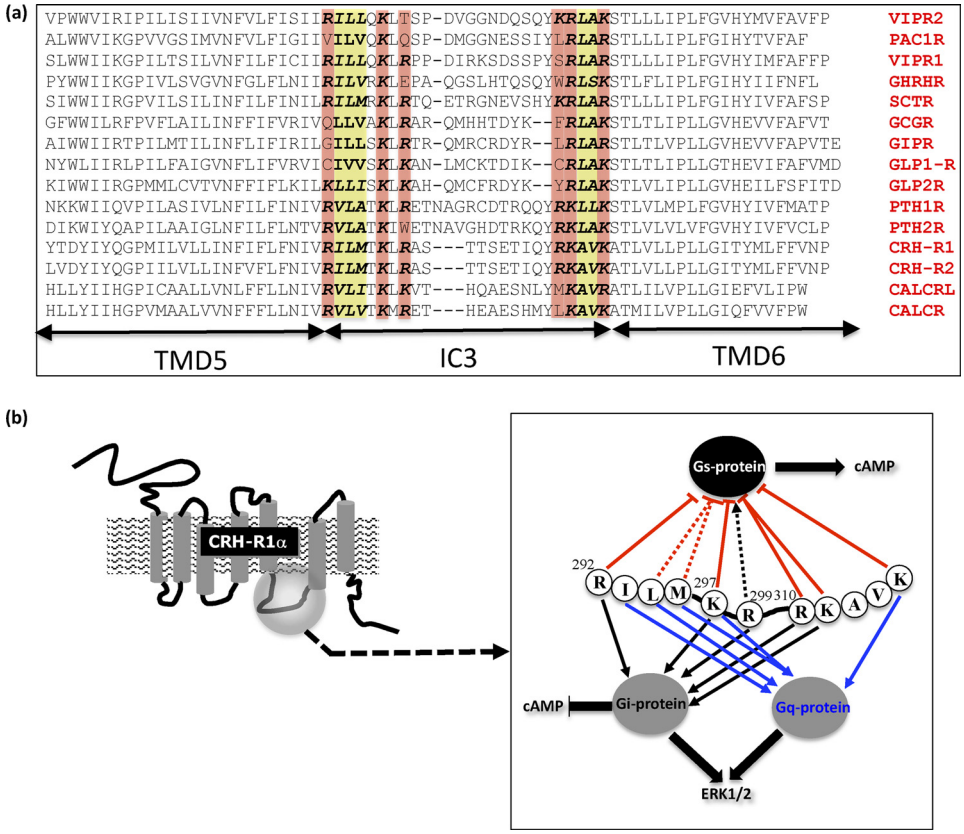


FIGURE 8. **Amino acid sequence alignment of family B1 GPCRs highlighting conserved motifs within IC3 and role of specific amino acids in CRH-R1α receptor signaling.** *a*, basic amino acids in the cationic α-helices B-I/L/V-L/V-*X* and B-A/L-*X*-B in the juxtamembrane regions near TMD5 and 6 highlighted in *red* and nonbasic conserved hydrophobic motifs in *yellow*. *b*, overview of amino acids within IC3 of CRH-R1α receptor identified as important for receptor G protein interactions and proposed roles in downstream cAMP and ERK1/2 signaling.

responses to PTX treatment identified a complex relationship and suggest that at high agonist concentrations IC3 mutant receptors might exhibit reduced coupling to PTX-sensitive $G_{i/o}$ proteins or alternatively increased activation of PTX-insensitive G proteins that exert an inhibitory action on AC activation (42). Previous studies (9) speculated that a possible receptor dimer formation might induce an allosteric inhibition of AC at high agonist concentrations.

The IC3 terminal segments were also shown to be important for receptor ability to induce ERK1/2 activation. Targeted disruption of the terminal cationic α-helices in mutants CRH-R1α(N-4A) and R1α(C-4A) led to a significant loss of agonist-induced ERK1/2 responses. Investigating the impact of Ala mutations on specific components of a complex mechanism that involves multiple G protein mediated pathways (4, 5, 28), we identified again Arg^{292}, Lys^{297}, Arg^{299}, Arg^{310}, and Lys^{311} as critical residues for PTX-sensitive G protein ($G_{i/o}$)-dependent ERK1/2 activation, and Ile^{293}, Leu^{294}, Met^{295}, Lys^{297}, and Lys^{314} as important for other pathways involved in ERK1/2 activation. In fact, the hydrophobic microdomain Ile^{293}-Met^{295} and the basic residues Lys^{297} and Lys^{314} present in the proximal and distal segments, respectively, appear to be critical structural determinants for the CRH-R1α-PTX-insensitive $G\alpha_q$ protein interaction that mediates phospholipase C activation and intracellular IP_3 accumulation. Basic residues within the juxtamembrane regions of IC3 have been shown to permit efficient coupling to G_q also for other class A and B GPCRs (15, 43). Recently, a polybasic motif in the C terminus of M3R was identified as critical for preassembly of M3 muscarinic acetylcholine receptors inactive state complexes with G_q, an interaction that increases the sensitivity and accelerates the onset of signaling (44).

In conclusion, the juxtamembrane regions of CRH-R1α contain microdomains crucial for receptor ability to differentially recognize and couple to distinct G proteins and downstream signaling. In particular, charged amino acids at either proximal or distal terminal segments play key roles for limiting G_s coupling and for activation of G_i proteins and thus provide a tight control of CRH-R1 signaling to prevent excess activity and hyperstimulation of the "stress" axis that might lead to pathological conditions.

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