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CASE

CRH-Stimulation of Cyclic Adenosine 5'-Monophosphate Pathway Is Partially Inhibited by the Coexpression of CRH-R1 and CRH-R2α

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Corticotropin-releasing hormone (CRH) is one of the major proteins responsible for brain stress regulation. Two well-known receptors have been described: type 1 and type 2α, both members of the receptor superfamily of G protein–coupled receptors (GPCR). We investigated receptor regulation when both CRH receptor subtypes are coexpressed in the same mammalian cell line. When both types of receptors are coexpressed, cAMP second messenger production is partially inhibited compared to when receptors are expressed separately. However, neither binding kinetics nor internalization rates are modified by coexpression of these receptors. To our knowledge this is the first demonstration of receptor interaction that results in the modification of CRH-mediated signal transduction pathway. Because CRH-R1 and CRH-R2α have overlapping mRNA expression patterns in the brain, these receptors may be coexpressed in neurons, suggesting that receptor interaction may play an important role in the effect evoked by CRH, contributing to the complexity of differential coupling of the CRH receptors in different endocrine and stress behavior responses.

Key Words: CRH receptors; cAMP pathway; receptor dimerization.

Introduction

Corticotropin-releasing hormone (CRH) is a 41-amino-acid peptide that stimulates the synthesis of adenocorticotrophic hormone (ACTH) and other products of propiomelanocortin (POMC) in the anterior pituitary *(1)*. Human and rat CRH are identical to one another and differ only by seven amino acids from ovine CRH. Two non-mammalian peptides similar to CRH, sauvagine (40 amino acids) and urotensin I (41 amino acids), share 50% homology with human/rat CRH *(2)*. CRH and urocortin I (UCN) bind with high affinity to CRH receptor type 1 (CRH-R1). Because CRH receptor type 2 (CRH-R2) binds urocortin with a 100-fold affinity in relation to CRH-R1, it was proposed that UCN was the endogenous ligand for the type 2 receptor; however, the limited overlap between the UCN neurons and the CRH-R2–expressing neurons suggested the existence of additional endogenous CRH-like peptides *(2)*. Two new members of the CRH family that bind exclusively to CRH-R2 have been described, stresscopin/urocortin III and stresscopin-related peptide/urocortin II *(3,4)*. The CRH receptor is a member of the class II of the superfamily of G protein–coupled receptors (GPCR). The effects that CRH evoke are mediated through at least two receptors: CRH-R1 *(5–7)*, and CRH-R2 *(8–10)*. CRH-R1 shares 70% homology with CRH-R2. CRH-R1 is highly distributed in the central nervous system, being the predominant receptor found in the corticotrophs in the anterior pituitary *(11,12)*. Distribution analysis of CRH-R2 showed that CRH-R2α is mainly localized in the brain while CRH-R2β is found in heart and skeletal muscles, brain, lung, and intestine *(9)*. Binding of CRH receptors to its specific agonists changes the CRH-R conformation, increasing the affinity of the receptor for the G_s protein, leading to the synthesis of cyclic AMP *(13)*. In addition, it has been demonstrated that CRH also activates the phospholipase C (PLC)-PKC signal transduction pathway in rat Leydig cells *(14)*, astrocytes culture *(15)*, and cerebellum *(16)*. Recently, it has been shown that in HEK-293 cell line stably transfected with the CRH receptors, both CRH-R1 and CRH-R2α are able to stimulate inositol phosphate (IP) production that accounts for the activation of Ca^{2+} transients by the direct activation of PLC in these cells *(17)*.

Several studies have demonstrated that some GPCR can form dimers. Heterodimer formation has been shown for β2-adrenergic *(18)*, δ-opioid *(19)*, and dopamine D_1, D_2, and D_3 receptors *(20–22)*, among others. Results obtained with those receptors indicate that dimerization affects the activation mechanism, as well as agonist-dependent desensitization and internalization *(18)*. Heterodimerization between receptors has also been described *(23–26)*.

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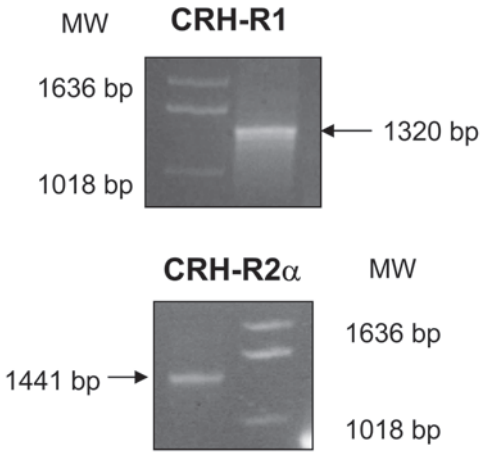

Fig. 1. RT-PCR of the cotransfected receptors in HEK-293 cells. In cells expressing both receptors, the RT-PCR experiment resulted in the amplification of two bands of 1320 bp and 1441 bp corresponding to CRH-R1 and CRH-R2α, respectively.

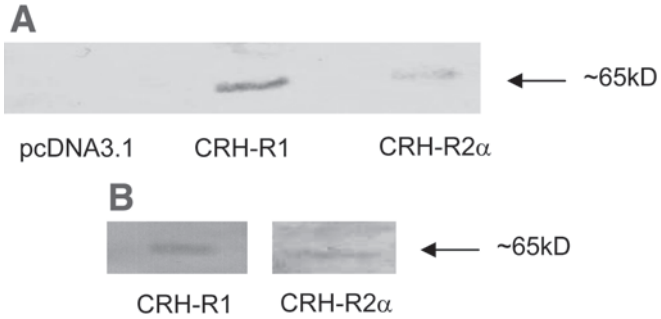

Fig. 2. Immunoprecipitation and Western blot analyses of CRH receptors. (**A**) HEK-293 cells transfected with CRH-R1 or CRH-R2α, and (**B**) HEK-293 cells transfected with both CRH-R1 and CRH-R2α. The receptors were immunoprecipitated from whole cells and identified by Western blot as described in Material and Methods.

Because CRH-R1 and CRH-R2α have overlapping mRNA expression patterns in the brain, suggesting that both receptors may be coexpressed in neurons, the aim of this study was to determine whether coexpression of CRH receptors modifies agonist binding, signal transduction, or internalization of CRH receptors. We found that coexpression of both receptor subtypes causes a significant reduction in the cAMP production compared with results obtained with cells expressing the receptors individually.

Results

CRH receptors were expressed either separately or collectively in HEK-293 cells. To confirm the presence of both receptors in the cells, CRH-R1 and CRH-R2α encoding mRNA was verified by RT-PCR. From total RNA obtained from these cells a band of 1320 bp was amplified using specific primers corresponding to CRH-R1 and a band of 1441 bp was amplified using specific primers for the CRH-R2α, respectively, in cells expressing both receptors (Fig. 1). No additional bands were observed. The presence of mature receptors was identified by immunoprecipitation and Western blot (Fig. 2). The results of immunoprecipitation and Western blot analyses revealed the presence of a 65 kDa band for each receptor when expressed separately (Fig. 2A), or coexpressed (Fig. 2B). We were unable to demonstrate the dimerization of the receptors by immunoprecipitation, but we cannot rule out the formation of CRH receptor dimers. In several studies where dimerization is assessed by coimmunoprecipitation, stringent solubilization might prevent the detection of constitutive dimers by promoting their partial or total dissociation.

Dose–response of CRH-stimulated cAMP production is shown in Fig. 3. Two hours of stimulation with CRH resulted in dose-dependent cAMP production from HEK-293 cells expressing CRH-R1, CRH-R2α, or CRH-R1 + CRH-R2α. Preliminary results in our laboratory (data not shown) determined that for cAMP production, the best amount of DNA transfected in HEK-293 cells was 0.1 μg/well of each receptor. A representative dose–response experiment of CRH-stimulated cAMP production is shown in Fig. 3. Experiments where concentration of one receptor was increased with respect to the other receptor to a final concentration of 0.1 μg/well are shown in Fig. 3A. A decrease in the cAMP production is observed when both receptors are present in the cells. Because the better response in terms of cAMP production was obtained when the concentration of each receptor was 0.1 μg/well, we decided to investigate the effect of 0.1 μg DNA of each receptor in the same transfection. When concentration of DNA is increased to a total of 0.2 μg, CRH-stimulated cAMP response with 10^{-7} *M* CRH showed a 70% decrease when compared with the response obtained by the CRH receptors separately (Fig. 3B). In order to determine direct interaction between receptors, the DNA complementation experiments were performed with the receptors and pcDNA3.1, and no decrease was observed in the cAMP response obtained in any transfection experiment (data not shown).

For the expression of CRH receptors, a two-binding-site model was the best fit for the binding results (Fig. 4). Scatchard analysis of the binding data showed that the dissociation constant (K_D) and *B*max for the high affinity sites for CRH-R1, CRH-R2α or CRH-R1 + CRH-R2α were 1.6 n*M* (*B*max 451,725 sites/cell); 0.5 n*M* (*B*max 266,217 sites/cell); and 1.54 n*M* (*B*max 458,350 sites/cell), respectively. The K_D and *B*max for the low affinity sites are 4.6 n*M* (*B*max 1,017,887 sites/cell), 8.5 n*M* (*B*max 1,746,670 sites/cell), and 8.2 n*M* (*B*max 1,638,256 sites/cell) for CRH-R1, CRH-R2α, or CRH-R1 + CRH-R2α, respectively.

Internalization rate data are shown in Fig. 5. Cells expressing CRH-R1 internalized a smaller percentage at steady state than the cells transfected with CRH-R2α and CRH-R1 + CRH-R2α. For all transfections the receptors are internalized at 30 min. The internalization rate before the cells

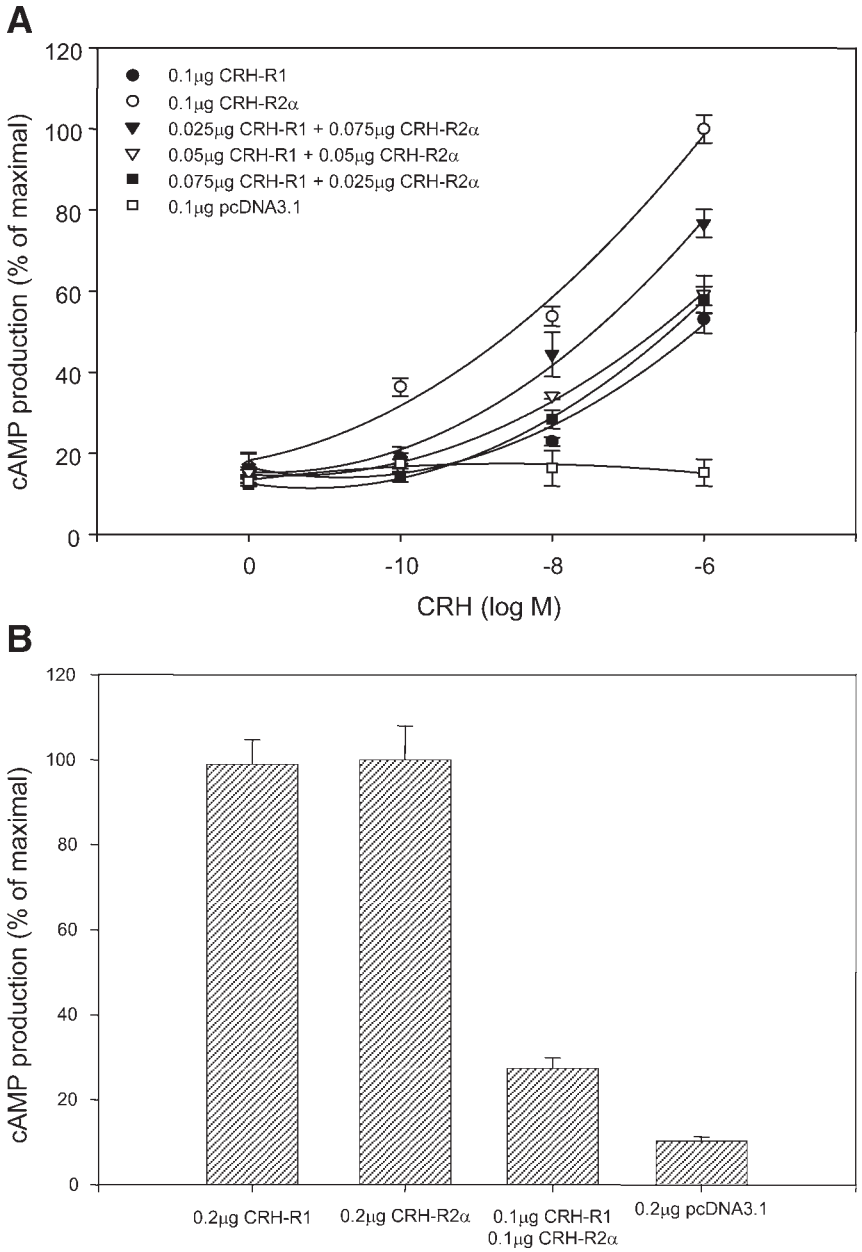

Fig. 3. cAMP production in cells cotransfected with the CRH receptors. (**A**) HEK-293 cells were transfected with the CRH receptors separately or cotransfected together at a final concentration of 0.1 μg of DNA, and the cAMP production was determined after the stimulation with different concentrations of CRH. (**B**) cAMP production was quantified after stimulation with 10^{-7} *M* CRH in cells transfected with CRH receptors at 0.2 μg final concentration of DNA. Each experiment was repeated at least three times with similar results.

reach a steady state are 0.56%, 0.48%, and 0.46% receptor internalized/min for CRH-R1, CRH-R2α, or CRH-R1 + CRH-R2α, respectively, indicating that the internalization rates are very similar between CRH receptors.

Discussion

This study shows for the first time that the coexpression of CRH-R1 and CRH-R2α significantly decreases the CRH-stimulated cAMP production in HEK-293 cells. Saturation bindings and Scatchard analysis showed that in all cases the agonist binding to its receptor fits in a two-binding-site model, with similar K_D and *B*max. After treatment with a CRH agonist, the receptors were internalized in a time-dependent manner and with similar internalization rates. The results obtained suggest that CRH-R1 and CRH-R2α may physically interact, modifying the functional characteristics of both receptors.

Our present results in the HEK-293 cells in which we observe an increased in the cAMP accumulation are similar to the well-known effects of CRH in the hippocampus, where the activation of specific receptors coupled to the G_s protein and to adenylate cyclase with a consequent increase in the cellular levels of cAMP *(27–30)*. It has been proposed

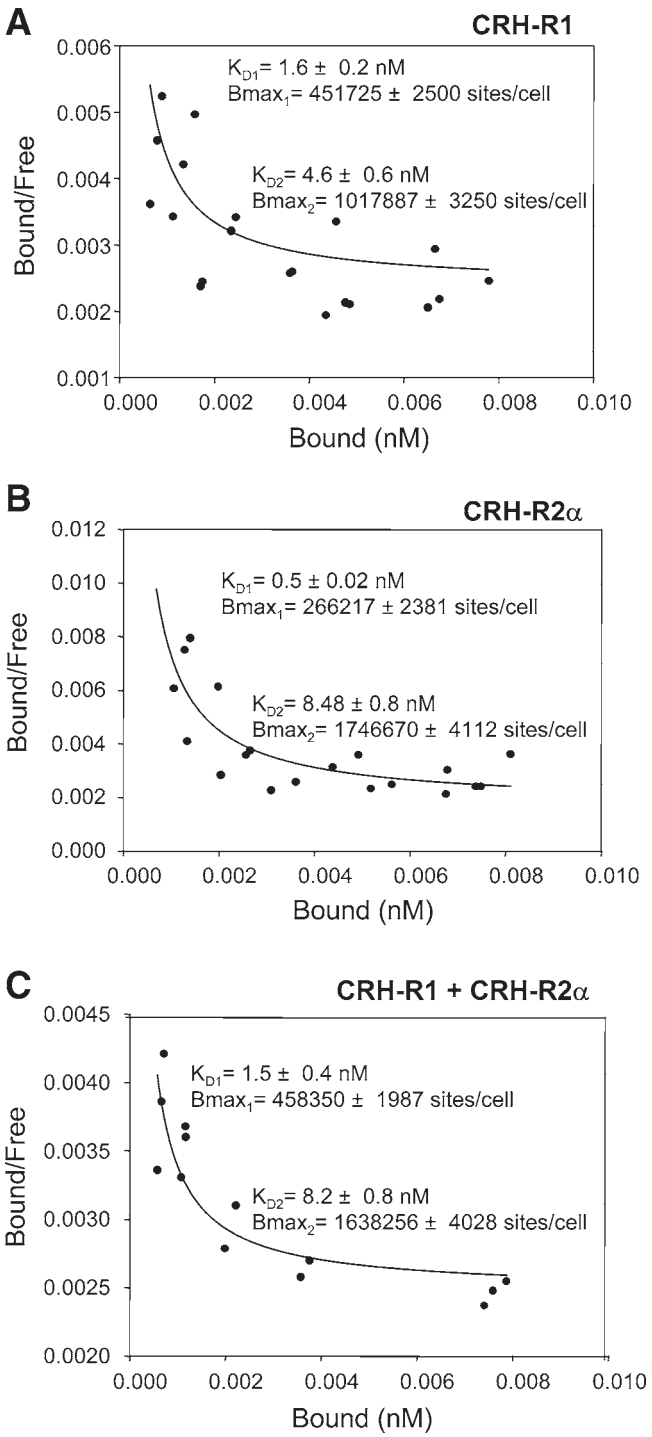

Fig. 4. Receptor binding of [^{125}I]CRH. Saturation binding of the HEK-293 cells expressing CRH-R1 (**A**), CRH-R2α (**B**), and CRH-R1 + CRH-R2α (**C**). Scatchard analysis demonstrated a two-binding-site model for CRH-Rs. The results are representative of three independent experiments.

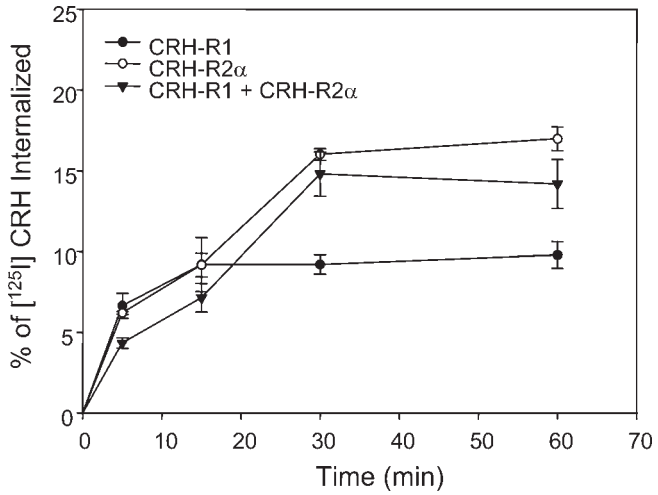

Fig. 5. Agonist induced CRH receptor internalization. HEK-293 cells were transiently transfected with CRH receptors were incubated with [^{125}I]CRH for the indicated times at 37°C. Cell-surface-bound and cytosolic-labeled ligand were determined as described in Materials and Methods. Each experiment was repeated at least three times with similar results.

that the coupling of CRH-R to a specific G protein depends on the cellular background, and the overexpression of the receptor can also induce the coupling to a different G protein; however, in the cell line we used we did not observed an increased in IP production (data not shown).

The coexpression of several GPCR in a single cell may potentially lead the formation of complex combination of homo- or heterodimers. It is possible that dimer formation is influenced by receptor expression levels and/or relative affinities of particular receptor combinations *(31)*. Evidence for the existence of GPCR dimerization has been widely reported indicating that this is a general mechanism of regulation for this receptor family *(31)*. The formation of dimers has been demonstrated for the somatostatin, opioid, angiotensin, GnRH, and β-adrenergic receptors, among others *(32–37)*. The heterodimerization between A_1 adenosine receptor and D_1 dopamine receptor uncoupled D_1 from activating adenylyl cyclase when both receptors are simultaneously activated *(38)*. Likewise, the coexpression of D_2 and D_3 dopamine receptors in a mammalian cell line displayed enhanced inhibition on adenylate cyclase when stimulated with both receptor ligands *(39,40)*. Coexpression of CRH-R1 and CRH-R2α partially inhibited cAMP response regulating negatively CRH-R signaling, while coexpression of either CRH-R1 or CRH-R2α with pcDNA 3.1 had no effect in signal transduction. When the receptors were cotransfected at 0.2 μg/well, the inhibition in the cAMP production was more evident. At this concentration we found the highest response of both receptors regarding the cAMP production when transfected independently or coexpressed. These results suggest an interaction between both types of receptors that account for the inhibition of the cAMP production; however, we cannot rule out that it may involve other interacting proteins that could be activated once the receptors are coexpressed and that is responsible for the decrease in the CRH signal transduction pathway. Because UCNII and UCNIII bind exclusively to CRH-R2α in further studies, it would be important to evaluate their

pharmacological effects in a CRH-R1/CRH-R2α coexpression system.

When cells coexpressed either CRH-R1 or CRH-R2α separately or together, receptor-ligand affinity does not change, suggesting that receptor interaction does not interfere with binding or expression of these receptors. Also, the Scatchard analysis indicated that in all expression combinations the [^{125}I]CRH was bound according to a two-site model of high- and low-affinity sites. Two affinity binding states for CRH-R1 and CRH-R2α has been reported previously in Y-93 cells *(41)*, in stably and transiently transfected HEK-293 cells *(42–45)*, and in rat pituitary and brain *(27)*. Previous studies performed with a non-hydrolyzable analog of GTP, GTPγS, showed that the high- and low-affinity components may indicate different degrees of receptor coupling to G proteins *(41)*. This result has been reported for TRHR1 and TRHR2, where the heterodimer displayed an altered internalization when compared to the individual receptors, although ligand binding was similar to when receptors expressed alone *(42)*. Similar results were obtained for the D2R homodimer, in which no change was observed in the binding properties for either agonists or antagonists *(43)*. It is also interesting that the K_D for the high-affinity site is similar to the K_D observed for the CRH-R1 alone, and that the K_D for the low-affinity site is similar to the K_D for the CRH-R2α alone.

After treatment with CRH, receptors were internalized in a time-dependent manner, but cells expressing CRH-R1 internalized a smaller percentage receptor at steady state; however, in all receptors the internalization rate is very similar. However, a discrepancy of our results with others studies is that CRH seems to activate the CRH-R2α receptor more. This may be explained because we used an heterologous system that does not naturally express either of the receptors.

The present results suggest that the coexpression of CRH-R1 and CRH-R2α substantially diminish CRH-stimulated cAMP production, but does not affect binding and internalization properties of these receptors. To our knowledge this is the first demonstration of receptor interaction that results in the modification of CRH-mediated signal transduction pathway. Because CRH-R1 and CRH-R2α have overlapping mRNA expression patterns in the brain, these receptors may be coexpressed in neurons, suggesting that receptor interaction may play an important role in the effect evoked by CRH, contributing to the complexity of differential coupling of the CRH receptors in different endocrine and stress behavior responses.

Material and Methods

Material

CRH and Tyr-CRH were purchased from Sigma-Aldrich Fine Chemicals (St. Louis, MO). pcDNA3.1, cell culture media Dulbecco's modified Eagle's medium (DMEM) and OPTI-MEM, lipofectamine, and PCR reagents and reagents for RNA extraction, TriZol and SuperScript Preamplification System were obtained from Invitrogen (Carlsbad, CA). Restriction enzymes, modified enzymes, competent cells, and the pGEM-T easy vector for subcloning were purchased from Promega (Madison, WI). Endofree Maxiprep kit was purchased from Qiagen (Valencia, CA). CRH-R1 (sc-1757) and CRH-R2α (sc-1826) antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The HEK-293 cell line was a kind gift from Dr. Ana María Pasapera-Limón, Research Unit in Reproductive Medicine, IMSS (Mexico, D.F.). cAMP antibody and protein A were kind gifts from Dr. P. Michael Conn, Oregon National Primate Research Center, OHSU (Beaverton, OR). All other reagents used were of the highest degree purity available from commercial sources.

CRH Receptor cDNA Cloning

Tissue was obtained from 28 d male Sprague–Dawley rats in accordance with the ethical committee of the Hospital de Pediatría del Centro Médico Nacional SXXI, IMSS. Total RNA was extracted from rat brain using TriZol reagent. Three micrograms of total RNA from rat brain was used to synthesize the cDNA using the SuperScript Preamplification System. Two primers were used in the PCR reaction: R1-1 [5'GACCGCAGCCGCCCGCCCTCCG3'] and R1-4 [5'GACTGGCTCGTCAGTGAGCTTGC3'], corresponding to the amino terminal region (from 1 to 21 bases) and the carboxyl-terminus (from 1375 to 1397 bases), respectively, from the CRH-R1 cDNA. Amplification of the cDNA was performed using Touchdown-PCR as previously described *(44)*.

The cDNA of the CRH-R2α receptor was obtained from the same tissue. RT-PCR and Touchdown PCR conditions were similar to those used in the CRH-R1 cloning. The primers used were R2A2 [5'CGCGCACTCCCACTCCCAA CGCGCG3'] corresponding to the region region 176/200 and R2A4 [5'GCACCCAAGGGTCAGTGTAGCAAG3'] corresponding to the region 1529/1551 of the CRH-R2α cDNA.

The PCR products were first subcloned into the pGEM-T easy vector. The wild-type cDNA for the CRH-R1 and CRH-R2α were further subcloned into pcDNA3.1 in the *Hind*III and *Xho*I restriction sites. The identity and the orientation of the cDNA cloning were confirmed by sequencing in an automated sequencer PE Applied Biosystem 373A (Perkin Elmer, Foster City, CA).

The identity of all constructs and the correctness of the PCR-derived sequences were verified by Dye Terminator Cycle Sequencing according to the manufacturer's instructions (Perkin-Elmer, Foster City, CA). For transfection, large-scale plasmid DNAs were prepared using a Qiagen Endofree Maxi-prep kit (Qiagen). The purity and identity of the amplified plasmid DNAs were further verified by restriction enzyme analysis.

Transient Transfections in HEK-293 Cells

Wild-type CRH receptors and altered receptors were transiently expressed in HEK-293 cells. This is an adherent human epithelial kidney cell line that does not express either of the CRH receptors. Cells were maintained in growth medium (DMEM) containing 10% fetal calf serum (FCS) and 20 μg/mL gentamicin in a 5% CO_2 humidified atmosphere at 37°C. Five thousand cells per well were seeded in six-well plates (Costar, Cambridge, MA). Twenty-four hours after plating, the cells were transfected with 2 μg DNA per well using 5 μL lipofectamine in 1 mL OPTI-MEM and 5 h later, 1 mL of DMEM containing 20% FCS calf serum was added to each well. Twenty-four hours after the start of transfection, the medium was replaced with fresh DMEM and the cells were allowed to grow for another 24 h before treatment.

Immunoprecipitation and Western Blot of the CRH Receptors

Transfected cells were washed with PBS prior to harvesting and homogenization in 500 μL of RIPA buffer (50 m*M* HEPES, pH 7.4, 150 m*M* NaCl, 1% Igelpal, 0.5% sodium deoxycholate, 1 m*M* EDTA, 2.5 m*M* $MgCl_2$) and protease inhibitor cocktail 1X (Pancreas-extract, pronase, thermolysin, chymotrypsin, trypsin, and papain; Roche, Germany). The homogenate was centrifuged for 10 min at 12,000*g* and 4°C. Cellular extracts were purified with 100 μL of protein A-Sepharose 6MB (Sigma-Aldrich Fine Chemicals) coupled to IgG from normal rabbit serum or normal goat serum for 30 min at 4°C. Furthermore, the remaining cellular extract was transferred to new tubes with 100 μL protein A-Sepharose pre-coupled to the CRH-R1 or CRH-R2α antibodies (2 mg of each antibody) and were incubated at 4°C overnight. Twelve hours later, the supernatant was discarded and the Sepharose were washed three times with RIPA buffer and the complex antibody–receptor was resuspended in 50 μL of SDS-PAGE buffer heating for 2 min at 95°C.

Samples were electrophoresed under reducing conditions on 10% polyacrilamide gels and electroblotted onto nitrocellulose membranes (Hybond C+, Amersham Pharmacia Biotech, Buckinghamshire, England). Bound antibody was detected using the horseradish peroxidase-conjugated goat anti-rabbit or mouse anti-goat immunoglobulin kit (BIO-RAD, Hercules, CA).

Quantification of cAMP Release

One hundred thousand HEK-293 cells were plated in a 24-well plate and transiently transfected with wild-type (WT) or altered receptor DNAs. Forty eight hours after transfection cells were washed twice with DMEM/0.1% BSA/20 μg/mL gentamicin and then stimulated with 0, 10^{-10}, 10^{-8}, or 10^{-6} *M* CRH in 500 μL of medium plus 0.2 m*M* methylisobutylxanthine (to inhibit phosphodiesterase activity) and were incubated for 2 h at 37°C. After stimulation, the medium from each well was collected in tubes containing 50 μL of 10 m*M* theophylline. The samples were heated at 95°C for 5 min and the amount of cAMP was determined by radioimmunoassay as described previously *(45)* in a gamma counter (PerkinElmer, Wellesley, MA).

Receptor Binding Assay

Intact cell CRH receptor binding was assessed over a range of 62,000–1,000,000 cpm of [^{125}I]CRH, prepared and purified as previously described *(46)*, in Tris-based binding buffer (50 m*M* Tris-HCl, pH 7.6, 100 m*M* NaCl, 2 m*M* $CaCl_2$, 5 m*M* KCl, 5% horse serum, and 0.5% fetal calf serum) DMEM/0.1% BSA. One hundred thousand cells were plated in a 24-well plate. Seventy-two hours after the start of transfection, cells were washed twice with Tris-based binding buffer. The radioactivity was added to each well and incubated at room temperature for 2 h. The cells were washed twice with Tris-based binding buffer and lysed with 1 *N* NaOH. The cell lysates were collected and the radioactivity was determined using a Wallac 10-channel gamma counter (Perkin Elmer).

For internalization studies, HEK-293 cells were transiently transfected using the same method as described above, except that 200,000 cells were plated per well in a 12-well plate. Approximately 72 h after transfection, the cells were washed twice with warm Tris-based binding buffer. The cells were incubated with [^{125}I]CRH (200,000 cpm) for the indicated times. At the appropriate time, the iodinated ligand was removed, and the plate was placed on ice. The cells were washed twice with ice-cold PBS and 500 μL of acid solution (50 m*M* acetic acid, 150 m*M* NaCl, pH 2.8) was added to each well and incubated for 12 min on ice. To determine the surface-bound iodinated ligand, the acid wash was collected and counted in a Wallac 10-channel gamma counter. To determine the internalized radioligand/receptor complex, cells were solubilized in 500 μL 0.1% Triton-X 100/PBS, collected, and counted. Nonspecific binding for all time points and cDNAs were determined using the same procedure but in the presence of 10 μ*M* unlabeled CRH. Nonspecific binding was substracted from the surface-bound and internalized radioligand and the internalized radioligand was expressed as the percentage internalized of the total bound at each time point.

The data shown represent the means ± SEM from triplicate incubations. In all experiments, the standard deviation was typically less than 10% of the corresponding mean. The data were analyzed by one-way ANOVA followed by the Duncan's multiple range test. $p < 0.05$ was considered statistically significant. Each experiment was repeated three or more times.

Acknowledgments

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