The corticotropin releasing factor (CRF) type 1 receptor (CRF₁) is a class B family G protein-coupled receptor that regulates the hypothalamic-pituitary-adrenal stress axis. Astressin is an amino-terminally truncated analog of CRF that retains high affinity binding to the extracellular domain of the receptor and is believed to act as a neutral competitive antagonist of receptor activation. Here we show that despite being unable to activate the CRF₁ receptor, astressin binding results in the internalization of the receptor. Furthermore, entirely different pathways of internalization of CRF₁ receptors are utilized following CRF and astressin binding. CRF causes the receptor to be phosphorylated, recruit βarrestin2 and to be internalized rapidly, likely through clathrin-coated pits. Astressin, however, fails to induce receptor phosphorylation or βarrestin2 recruitment, and internalization is slow and occurs through a pathway that is insensitive to inhibitors of clathrin-coated pits and caveolae. The fate of the internalized receptors also differs since only CRF-induced internalization results in receptor downregulation. Furthermore, we present evidence that for astressin to induce internalization it must interact with both the extracellular amino terminus and the juxtamembrane domain of the receptor. Astressin binds with 6-fold higher affinity to full length CRF₁ receptors than to a chimeric protein containing only the extracellular domain attached to the transmembrane domain of the activin IIB receptor, yet two 12-residue analogs of astressin have similar affinities for both proteins but are unable to induce receptor internalization. These data demonstrate that agonists and antagonists for CRF₁ receptors promote distinct conformations, which are then differentially regulated.

The 41 amino acid neuropeptide corticotropin releasing factor (CRF) is the principal regulator of the hypothalamic-pituitary-adrenal axis, and as such plays a critical role in mediating the body’s response to stress(1,2). In mammals, CRF and the related urocortins (UCN) 1, 2 and 3 bind to and activate two distinct G protein-coupled receptors (GPCRs), termed CRF₁ and CRF₂(3). The CRF₁ receptor is expressed mainly in the pituitary and central nervous system, where it is responsible for most of the central functions of CRF and UCN 1, including integration of endocrine, autonomic and behavioral responses to stress, and adrenocorticotropic hormone (ACTH) release from corticotrope cells of the anterior pituitary(4). Furthermore, there is strong evidence that alterations in the CRF₁ receptor system occur in many anxiety and depressive disorders(5-7). CRF₂ receptors bind all three UCNs with high affinity and CRF with lower affinity(3). These receptors exist as three independent isoforms (CRF₂(a), CRF₂(b) and CRF₂(c)) and are expressed both in the central nervous system and the periphery, including in the heart, skeletal muscle, gastrointestinal tract and epididymis(3). The functions performed by the various isoforms of the CRF₂ receptor are currently being elucidated(8). Both CRF₁ and CRF₂ receptors belong to the Class B family of G protein-coupled receptors, which includes (but is not limited to) the receptors for glucagon, parathyroid hormone (PTH), secretin.
and vasoactive intestinal peptide. All class B receptors possess a large extracellular domain (ECD) with which they bind with high affinity to the carboxyl terminal regions of their peptide ligands(9). This interaction alone is not sufficient to stimulate coupling of the receptor to G proteins, however, and a second interaction must occur between the juxtamembrane domain of the receptor (the transmembrane helices and intervening loops) and the first few residues within the amino terminal portion of the peptide ligand(7,10). Since discrete regions of class B ligands perform high affinity binding and receptor stimulation, truncating the endogenous peptides at their amino termini produces high affinity competitive antagonists for class B receptors. Further modifications made to CRF truncated in this manner have produced a number of different antagonist peptides including astressin (cyclo (30-33)[D-Phe12, Nle21,38, Glu30, Lys33]CRF(12-41)), a high affinity antagonist for CRF1 receptors that also possesses enhanced biological stability, allowing its extensive use in vivo to dissect the functions of the CRF system(11,12). Astressin has no detectable agonist activity at the CRF1 receptor and thus is believed to act as a neutral competitive antagonist. In addition to binding to the ECD of CRF1 receptors, a recent report has suggested astressin may form a second low affinity contact with the juxtamembrane domain since astressin retains the ability to inhibit CRF activation of a CRF1 receptor fragment that lacks the ECD(13).

Following activation by agonists, almost all GPCRs undergo a series of modifications to prevent continuous signaling of the receptor, and to enable the cells on which they are expressed to regulate their sensitivity to future exposures to agonist. This is achieved first by preventing the activated receptors from further interacting with G proteins (desensitization), and then by internalizing the receptors into intracellular compartments (also called sequestration or endocytosis) (14,15). Desensitization occurs through phosphorylation of intracellular domains of the receptor by GPCR kinases (GRKs) that specifically recognize agonist-occupied receptor molecules, followed by the recruitment and binding of βarrestins, which sterically hinder further receptor-G protein coupling. The subsequent internalization of the receptors can occur through multiple pathways, the most common of which utilize clathrin-coated pits and caveolae, although some less well-defined pathways have also been described, including those that use non-coated vesicles and macropinosomes(16). Internalization can result in either short or long-term reductions in sensitivity to further agonist stimulation depending on whether the receptors become resensitized and recycle back to the cell surface, or are targeted for degradation (downregulated)(17).

A few examples of GPCRs undergoing regulation by antagonists have also been described, including internalization and downregulation of the 5HT2A receptor by several atypical antipsychotics(18,19); downregulation of the gonadotropin releasing hormone receptor (GnRHR) in pituitary gonadotrophs by the GnRH analogue cetrorelix(20); and phosphorylation and internalization of angiotensin II type 1A receptor (AT1A) by several antagonist peptide analogs of angiotensin II(21,22). Furthermore, it has recently been reported that the class B PTH1 receptor also undergoes internalization following binding to the truncated antagonist peptide PTH(7-34), a process that is independent of receptor activation (23). In light of these discoveries, we investigated whether this phenomenon of peptide antagonist-induced internalization also occurs with CRF1 receptors, and to probe the mechanism(s) underlying this process.

Methods

Materials: Peptides were synthesized by solid-phase methodology on a Beckman Coulter 990 peptide synthesizer (Fulton, CA) and purified as previously described (13). All chemicals were purchased from Sigma-Aldrich unless otherwise stated. Tissue culture medium and reagents were from Mediatech (Herndon, VA), except fetal bovine serum (FBS) from HyClone (Logan, UT) and horse serum from Invitrogen Life Technologies (Carlsbad, CA). Renilla mulleri GFP (RmGFP) was licensed from Prolume Inc. (Pinetop, AZ). Membranes prepared from Ltk- cells expressing human CRF1 (hCRF1) receptors and from human embryonic kidney (HEK-293) cells expressing hCRF1, rat CRF1 (rCRF1) and rCRF1-ECD/activin IIB chimera receptors have been described previously(13,24).
Mammalian expression constructs: Construction of hCRF₁ receptor tagged with the hemagglutinin signal sequence and FLAG epitope (HA-FL-CRF₁) in pcDNA5/FRT/V5-His<sup>E</sup>TOPO®<sup>©</sup>, its stable expression in CHO-K1 Flp-In cells (designated CHO-CRF₁ cells), and its indistinguishable pharmacology from the wild-type hCRF₁ receptor are described previously<sup>(25)</sup>. Complementary DNAs for dynamin1, caveolin1 and β-arrestin2 were amplified from a human brain cDNA library, and inserted into the pcDNA3.1/V5-His<sup>E</sup>TOPO® vector following the manufacturer’s instructions. Mutations were made in dynamin1 (K44A) and caveolin1 (S80A, S80E)<sup>(26)</sup> using the Quickchange<sup>®</sup> site-directed mutagenesis kit following the manufacturer’s instructions (Stratagene, La Jolla, CA). The β-arrestin2<sub>Rm</sub>GFP construct was made by adding EcoRI restriction sites to the β-arrestin2 and RmGFP coding sequences by PCR with the primer pairs 5'-AAAGAATTCCACATGGGGAGAAAACC-3', 5'-AGAATTCGGAAGCAAGCAGATCCTGAAGAATCC-3', and 5'-AAAGAATTCCGAGAGTTGATCATCATAGTCACCTGAAGAGAGAAAACC-3', respectively. Both products were cloned into pcDNA3.1/V5-His<sup>E</sup>TOPO® and subsequently digested with EcoRI. The released β-arrestin2 fragment was purified and subsequently ligated into the linearized RmGFP construct. All plasmid DNA constructs were amplified in E.coli using standard molecular biology procedures, harvested using Qiagen<sup>®</sup> DNA preparation kits, and their correct sequences confirmed by DNA sequence analysis using an ABI377 automated DNA sequencer and BigDye™ Terminator v3.0 sequencing kits (Applied Biosystems, Foster City, CA).

Cell culture and transfection: HEK-293 cells, Chinese hamster ovary Flp-In (CHO-K1 Flp-In) cells and mouse pituitary corticosterone adenoma AtT-20/D16v-F2 cells (AtT20, purchased from ATCC) were maintained at 37°C and 5% CO₂ in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10mM HEPES pH 7.4, 0.2mM glutamine, 1mM sodium pyruvate, and penicillin-streptomycin (50I.U./ml and 50µg/ml, respectively), and either 10% (v/v) heat-inactivated fetal bovine serum (FBS) for HEK-293 and CHO-K1 Flp-In, or 10% (v/v) heat-inactivated horse serum for AtT20. Stable expression of receptor in CHO-CRF₁ cells was maintained by selection with 500µg/ml hygromycin B. Transient transfections into HEK-293 cells were performed using Fugene6<sup>®</sup> (Roche, Indianapolis, IN) at a ratio of 3µL Fugene6<sup>®</sup> to every 1µg plasmid DNA. Experiments were performed on these cells 48 hours after transfection. Overexpressed caveolin1 mutants and dynamin1 K44A were detected in cell lysates separated by SDS-PAGE, transferred to nitrocellulose and immunoblotted with anti-caveolin1 and anti-dynamin1 antibodies (Upstate, Charlotteville, VA), followed by incubation with hors eradish peroxidase-conjugated secondary antibodies (Amersham Biosciences, Piscataway, NJ) and detection by chemiluminescence (Pierce, Rockford, IL).

Measurement of receptor internalization by flow cytometry: Cells transiently or stably expressing FL-CRF₁ receptor were seeded at 4x10<sup>5</sup> cells/well in poly-D-lysine-coated 6-well dishes (BIOCOAT™, Fort Washington, PA). The following day the cells were subjected to the appropriate drug treatments, washed twice with ice-cold internalization medium (DMEM containing 25mM HEPES pH 7.4, 0.2mM glutamine, 1mM sodium pyruvate, 50I.U./ml penicillin and 50µg/ml streptomycin) and then incubated for one hour at 4°C with anti-FLAG M2 antibody (Sigma-Aldrich, St. Louis, MO) diluted 1:500 in internalization medium. The cells were then washed three times with ice-cold internalization medium and incubated at 4°C in the dark for a further 30 minutes with goat anti-mouse IgG antibody conjugated to AlexaFluor<sup>TM</sup>488 dye (Molecular Probes, Eugene, OR) diluted to 1:250 in internalization medium. Cells were subsequently washed with ice-cold PBS three times, detached from the dishes with PBS containing 5mM EDTA and fixed by the addition of formaldehyde to 0.8% w/v. The fluorescence intensity of 10<sup>4</sup> cells from each well was then measured on a FACScan™ flow cytometer (BD Biosciences, San Jose, CA). Concanavalin A (Sigma) was added to the cells at 0.25mg/ml 1 hour prior to stimulation. Hypertonic medium treatment, potassium depletion and disruption of caveolae with filipin III were carried out using previously described methods<sup>(27-29)</sup>.
Examination of Receptor internalization by Fluorescence Microscopy: CRF₁ receptor internalization was visualized using a previously described method with a minor modification (30). Briefly, transiently transfected AtT20 or HEK-293 cells expressing FL-CRF₁ receptor with or without β-arrestin2-RmGFP were grown on Nunc® Lab-Tek® II CC2® multichamber glass slides. Surface receptors on AtT20 cells were labeled (20 minutes at 37°C) with AlexaFluor™488-conjugated M1 anti-FLAG antibody, or on HEK-293 cells with M1 anti-FLAG-Cy3 conjugate (prepared according to the manufacturer’s instructions using either Alexa Fluor™488 Monoclonal Antibody Labeling Kit, (Molecular Probes), or Cy3 monoreactive Dye Pack (Amersham Biosciences)). Cells were washed once and exposed to 10 µM CRF or 10 µM astressin (2 hours at 37°C) to induce internalization. Cells were washed twice with PBS and immediately fixed with 4% w/v paraformaldehyde in PBS for 15 minutes at room temperature. Cells were washed three times at 10 minute intervals with PBS before mounting using ProLong® Gold antifade reagent (Molecular Probes). Fluorescence was visualized on an Olympus IX70 inverted microscope equipped with the CARV confocal module (Kinetic Imaging, Nottingham, UK) using appropriate dichroic filter sets. Images were acquired with a MicroMAX cooled CCD camera (Princeton Instruments, Trenton, NJ) and processed using MetaMorph® Imaging System (Universal Imaging Corporation, Downingtown, PA).

[^32P]orthophosphate ([^32P] labeling and receptor immunoprecipitation: CHO-CRF₁ or CHO-K1 Flp-In cells were serum starved for 1 hour in phosphate-free DMEM supplemented with 2 mM L-glutamine and 25 mM HEPES pH 7.4. Cells were labeled with 100 µCi/ml of [^32P] (PerkinElmer, Wellesley, MA) for 1 hour, then 100 nM microcystin-LF (Calbiochem, San Diego, CA) was added and the cells incubated for a further 15 minutes before stimulation with 100 nM astressin or CRF at 37°C, or left untreated. Cells were washed twice with PBS and extracts were prepared by lysing cells in 500 µL glycerol lysis buffer (50 mM HEPES pH 7.4, 0.5% v/v NP-40, 250 mM NaCl, 2 mM EDTA, 10% v/v glycerol, 100 µM NaVO₃, 10 mM NaF, 100 mM microcystin-LF, and Complete® EDTA-free protease inhibitor cocktail tablet (Roche)). The samples were clarified by centrifugation, and FL-CRF₁ receptor was immunoprecipitated from equal amounts of cell lysate with 30 µL M2 anti-FLAG-agarose conjugate (Sigma-Aldrich) for 16 hours with constant mixing. Immunoprecipitates were washed 5 times with glycerol buffer and eluted with 40 µL 2X SDS sample buffer (Invitrogen) supplemented with 200 mM DL-1,4-dithiothreitol. Half the volume (20 µL) of the immunoprecipitates was resolved on a 4-20% Tris-Glycine SDS-polyacrylamide gel (Invitrogen). The gel was dried and incorporation of [^32P] was measured using the VersaDoc3000 phosphorimager (Bio-Rad, Hercules, CA).

Measurement of receptor downregulation by ELISA: CHO-CRF₁ cells were seeded at 7.5x10⁴ cells/well in poly-D-lysine-coated 96-well dishes (BIOCRAITM, Fort Washington, PA). The following day the cells were subjected to the appropriate treatments, washed once with ice-cold PBS and lysed in 200 µL of ice-cold lysis buffer (1% v/v NP-40 in PBS supplemented with Complete® EDTA-free protease inhibitor cocktail, Roche) for 30 minutes at 4°C with constant agitation. Detergent insoluble fractions were sedimented by 10 min centrifugation at 2,000xg, and the amount of CRF₁ receptor present in the clarified cell lysates was quantified by ELISA. Briefly, protein concentration was measured using the BCA protein assay method (Pierce, Rockford, IL). Equal amounts of protein (12 µg/well) were transferred to anti-FLAG M2 coated 96-well plates (Sigma-Aldrich, St. Louis, MO) and incubated overnight at 4°C. Each well was washed 4 times with ELISA wash buffer (0.05% v/v Tween-20 in PBS), incubated for 2 hours at room temperature with the previously described anti-CRF₁ receptor antiserum 4467a-CRF₁ (31) diluted 1:10,000 in antibody dilution buffer (PBS containing 1% w/v BSA). The plates were washed, incubated for 1 hour at room temperature with horseradish peroxidase-conjugated anti-rabbit IgG (Amersham Biosciences) diluted 1:2,000 in antibody dilution buffer (PBS containing 1% w/v BSA). The plates were washed, incubated for 1 hour at room temperature with horseradish peroxidase-conjugated anti-rabbit IgG (Amersham Biosciences) diluted 1:2,000 in antibody dilution buffer, followed by a final four washes. Plates were incubated with 200 µL/well of ready-to-use horseradish peroxidase substrate 3,3’,5,5’-tetramethylbenzidine (Sigma-Aldrich) for 30 minutes at room temperature, followed by addition of 100 µL/well 0.5M H₂SO₄ to stop the reaction.
The optical density of each well was read at 450nm using an EMax microplate reader (Molecular Devices, Sunnyvale, CA).

RESULTS

To determine whether CRF and the antagonist astressin are both capable of inducing the internalization of the CRF1 receptor, CHO-CRF1 cells (CHO-K1 cells stably expressing the HA-FLAG-tagged hCRF1 receptor) were treated with 100nM CRF or astressin for between 30 minutes and 24 hours, and the loss of receptors from the cell surface was measured by flow cytometry. Time-matched vehicle-treated controls were also performed to allow all data points to be normalized to the appropriate level of cell surface expression. Figure 1A shows that both CRF and astressin induced substantial internalization over this period, however the total amount of internalization following 24 hours stimulation with CRF was greater than with astressin (71±7.4% and 51±8.1%, respectively). This difference was the result of a higher rate of receptor sequestration by CRF in the first hour of stimulation, (63% with CRF, 17% with astressin), after which sequestration by both peptides proceeded at similar rates (evident from the similar slopes of the graphs between 2 and 24 hours in Fig. 1A). Full dose-response relationships were then produced for CRF and astressin to allow the EC50 values to be calculated (Fig 1B). Both peptides internalized CRF1 receptor in a dose-dependent manner, however, despite the fact that astressin possessed only partial efficacy in the internalization assay, the redistribution of the immunofluorescence-stained receptors was monitored. No internalization of CRF1 receptors was observed in cells treated with vehicle for 2 hours (Fig. 2Ai), demonstrating that the conjugated M1 antibody alone did not induce receptor internalization. Following treatment with CRF, however, many of the labeled receptor molecules had redistributed from the cell surface into compartments within the cytosol (Fig. 2Aii). A less robust but similar pattern of CRF1 receptor redistribution to intracellular compartments was observed following astressin treatment, with substantial levels of receptor remaining at the cell surface (Fig. 2Aiii). This reduced level of receptor redistribution with astressin correlated well with the amount of CRF1 receptor internalization measured in AtT20 cells using flow cytometry (Fig. 2B), where CRF induced internalization of 39% after 2 hours, increasing to 45% at 4 hours, while astressin internalized 9% after 2 hours and 16% after 4 hours.

We next determined whether the phenomenon of astressin-induced internalization also occurred in a cell line that expresses the CRF1 receptor endogenously. Corticotropes of the anterior pituitary are major sites of CRF1 receptor expression, where it stimulates the secretion of ACTH into the blood in response to CRF released from the hypothalamus(2,32). The mouse pituitary corticotrope adenoma AtT20 cell line expresses CRF1 receptors (~100fmol/mg membrane protein, data not shown), produces cyclic AMP(25) and secretes ACTH(33) when challenged with CRF peptide. To measure CRF1 receptor internalization, AtT20 cells were transfected with FLAG-tagged CRF1 receptor to allow its movement to be tracked both by immunocytochemistry and by flow cytomtery (Fig. 2). Prior to treatment, immunostaining of live AtT20 cells with anti-FLAG M1 antibody conjugated to AlexaFluor™488 dye revealed substantial cell surface expression and no visible staining of intracellular receptors (not shown). The cells were then treated for 2 hours with vehicle, or with a maximal dose of CRF or astressin (10µM), and the redistribution of the immunofluorescence-stained receptors was monitored. The majority of GPCRs are internalized via clathrin-mediated endocytosis(16). This process normally requires the receptors to be bound to agonist, phosphorylated by GRKs and to recruit cytosolic arrestins, however, clathrin-mediated internalization following antagonist binding has also been reported(34). Furthermore, some peptide antagonists are known to cause receptor phosphorylation without activating G protein coupling(21,22,34). To determine if such mechanisms could underlie astressin-induced CRF1 receptor internalization, we compared the ability of astressin and CRF to promote CRF1
receptor phosphorylation and the recruitment of β-arrestin2 (Fig. 3A and 3B). CHO-CRF1 cells were metabolically labeled with $^{32}$P$i$, stimulated with 100nM astressin or CRF for between 5 minutes and 1 hour, and the amount of radioactivity incorporated into the CRF1 receptor was assessed by autoradiography of receptor immunoprecipitates. Figure 3A shows that stimulation of cells with astressin caused no phosphorylation of the receptor at any of the time points tested. In contrast, stimulation with CRF induced robust phosphorylation of CRF1 receptors by 5 minutes (4.27±0.28-fold over basal), peaking at 10 minutes (4.46±0.54-fold over basal) and then slowly diminishing to 2.08±0.54-fold after 1 hour. Next, we tested whether stimulation with astressin or CRF led to recruitment of cytosolic β-arrestin2 to CRF1 receptors. Cells were first transfected with expression constructs for β-arrestin2-RmGFP and FL-CRF1 receptor. Forty eight hours later, receptors expressed at the cell surface were visualized by immunostaining with anti-FLAG M1 antibody conjugated to Cy3, then the cells were stimulated with 100nM astressin or CRF for 5 or 15 minutes, and the distributions of receptor and β-arrestin2 were determined by fluorescence microscopy (Fig. 3B). Prior to stimulation, the CRF1 receptor was only detected on the cell surface, while β-arrestin2-RmGFP was evenly distributed throughout the cytosol and excluded from the nucleus. After 5 minutes of stimulation with CRF, the receptors were still present at the cell surface, however the majority of the β-arrestin2-RmGFP had localized to the cell membrane and displayed a distribution that almost completely overlapped with the CRF1 receptor. After 15 minutes, much of the receptor and β-arrestin2-RmGFP had redistributed from the cell surface into punctate structures within the cytosol, again showing almost complete overlap of their distributions. This was entirely different from what was observed following astressin stimulation: no relocation of β-arrestin2-RmGFP was observed after 5 or 15 minutes stimulation, while a small amount of receptor internalization could be observed after 15 minutes in some cells (Fig. 3B). Taken together, these data demonstrate that CRF binding to the CRF1 receptor triggers phosphorylation of the receptor, recruitment of β-arrestin2 and internalization of receptor-β-arrestin2 complexes, while astressin treatment induces neither phosphorylation nor β-arrestin2 recruitment, but still induces receptor internalization.

Next, we sought to identify the mechanisms by which CRF and astressin induced CRF1 receptor endocytosis by testing their sensitivities to known inhibitors of internalization pathways. Clathrin-mediated endocytosis is sensitive to both treatment with hypertonic medium (DMEM containing 0.5M sucrose(27)), and to potassium ion depletion(28). CHO-CRF1 cells were subjected to both these treatments prior to the addition of 100nM CRF or astressin for 1 hour, and internalization was measured by flow cytometry (Fig. 3C and 3D). CRF-induced receptor internalization was strongly inhibited by both sucrose (83%), and potassium depletion (97%), the latter effect being largely reversed when potassium was replaced in the depletion buffer (compare 34.5±4.7% with potassium replacement to 47.4±2.4% when cells were maintained in medium). In contrast, neither of the treatments significantly affected astressin-induced internalization indicating that, while CRF-driven endocytosis is likely a clathrin-mediated process, astressin utilizes an entirely different endocytic pathway. Several GPCRs have been reported to internalize through caveolae, cup-like cholesterol-rich membrane structures that contain large quantities of the membrane-associated protein caveolin1 and, like clathrin-coated pits, require dynamin1 to internalize receptors(35,36). Three approaches were taken to determine if caveolae were utilized by astressin or CRF to internalize CRF1 receptors. Firstly, CHO-CRF1 cells were treated with the cholesterol-depleting agent filipin III under conditions previously demonstrated to cause disruption of most of the caveolae in cells (1µg/ml filipin III for 1 hour(29)), and then receptor internalization was measured after 1 hour of stimulation with 100nM astressin or CRF. Filipin III failed to inhibit internalization induced by either peptide, suggesting caveolae were not involved (Fig. 3E). Secondly, overexpression of the dominant negative mutant dynamin1 K44A, which is required for both caveolae- and clathrin-mediated endocytosis, failed to inhibit astressin-induced internalization but, as was expected, did significantly reduce internalization by CRF (Fig. 3F). Thirdly, coexpression of CRF1 receptors with
the dominant negative S80E mutant of caveolin1 (or the phenotypically neutral S80A mutant)(26) failed to disrupt either astressin- or CRF-induced receptor internalization (Fig. 3F). Taken together, these data indicate that cells treated with astressin utilize neither caveolae nor clathrin-coated pits to internalize CRF1 receptors, while CRF-induced internalization likely occurs through clathrin-coated pits, as demonstrated by its sensitivity to potassium depletion, hypertonic sucrose solution and K44A dynamin expression.

Following internalization many GPCRs are downregulated by trafficking to lysosomes for degradation, while others remain sequestered within intracellular compartments(17). We tested whether the use of different internalization pathways following CRF and astressin stimulation also resulted in alternative trafficking and processing of the CRF1 receptor. An ELISA detection method was developed to measure loss of receptor protein in cell lysates at between 1 and 24 hours following stimulation with either 100nM astressin or CRF. Figure 4A shows that there was no reduction in total CRF1 receptor protein following astressin stimulation at any of the time points tested, while stimulation with CRF showed a gradual loss of the receptor in the cells, from 83% remaining after 1 hour, dropping to 38% after 24 hours. Despite the kinetics of receptor downregulation being markedly different from those we observed for receptor internalization (Fig. 1A, and shown on Fig. 4A for comparison), the downregulation is dependent on CRF1 receptor internalization since cross-linking of the receptor on the cell surface with the lectin concanavalin A prior to stimulation suppressed internalization by both astressin (53% inhibited) and CRF (55% inhibited) and also inhibited CRF-stimulated downregulation of the receptor by 53% (Fig. 4B).

We next investigated the nature of the interactions between astressin and the receptor that are required for receptor internalization. Peptide interaction with CRF1 receptors proceeds according to a two-domain model in which the carboxyl terminal portion of the ligand binds the ECD, and the amino terminal portion binds the juxtamembrane domain. In addition to the high affinity interaction with the ECD, it was recently reported that astressin might also interact with low affinity with the juxtamembrane domain(13). For example, astressin bound with higher affinity to the full-length receptor than to a chimera of the ECD and the single transmembrane domain of the activin IIB receptor, suggesting a second astressin binding site within the juxtamembrane domain. This raises the possibility that an interaction of astressin with the juxtamembrane domain is involved in receptor internalization. This hypothesis was tested using 12-residue carboxyl terminal astressin analogs that have been reported to bind CRF1 receptors with high affinity (Yamada #19 and Yamada #20(37)). These peptides lack 18 amino terminal residues of astressin and so would be predicted to bind only the ECD, according to the two-domain model described above. Indeed, both peptides bound with similar, if not slightly higher, affinity to the rat CRF1-ECD/activin IIB chimera than to the full-length receptor, consistent with these peptides binding predominantly, if not exclusively to the ECD (Table 1; since our previous experiments were conducted using hCRF1 receptors, we also confirmed the affinities of all three peptides were similar for both rat and human forms). In contrast astressin bound with 6-fold higher affinity to the full-length receptor than the CRF1-ECD/activin IIB chimera, suggesting that binding is stabilized by interaction with the juxtamembrane domain (Table 1, consistent with previous data(13)). Yamada #19 and Yamada #20 bound with high affinity to hCRF1 receptors (Fig. 5A, Table 1, $K_i = 1.3nM$ and 1.0nM respectively, in agreement with published values(37)). Full internalization dose-response relationships of both peptides were then compared to those for CRF and astressin and the EC50 values were calculated (Fig. 5B). Interestingly, while CRF and astressin potently induced internalization of the CRF1 receptor (EC50 of 5nM and 1nM, respectively), neither of the Yamada peptides showed any activity in this assay at concentrations up to 10µM (Fig. 5B). These findings suggest that interaction with the ECD alone is insufficient to induce internalization. Furthermore, the structure-activity relationship of these ligands suggests that internalization involves interaction of the amino terminal region of astressin with the juxtamembrane domain of the receptor.

**DISCUSSION**

It is now widely recognized that most GPCRs are internalized following their stimulation
CRF₁ Receptor Internalization By Antagonists

with ligand, a process that prevents their persistent signaling and allows cells to regulate receptor number, and thus sensitivity to subsequent challenges with agonist. Internalization, coupled with receptor desensitization, is also an important process in the pathogenesis of several diseases, the development of tolerance to certain drugs, and may contribute to the efficacy of other therapeutics. For example, loss of β-adrenergic receptors from cardiac myocytes leads to heart disease(38), while desensitization of opioid receptors is believed to underlie tolerance to opiates(39), and at least part of the therapeutic activity of cetorelix is a result of its ability to downregulate the GnRH receptor(20). The first two examples represent the well-studied “classic” mechanism of GPCR regulation following stimulation (or over-stimulation) with a native or synthetic agonist. The last example, however, is more interesting since it results from the binding of an antagonist to the receptor, causing the receptor to become downregulated without it ever being activated. Since current models of receptor regulation involve a priori activation of the receptor, the existence of such antagonist-mediated mechanisms has profound implications for our present understanding of how GPCRs function and are regulated, and possibly for interpreting the in vivo activities of these compounds.

In this study we describe a previously unrecognized mechanism by which cells regulate CRF₁ receptors following binding to the antagonist peptide astressin, a process that promotes endocytosis of the receptor molecules and their subsequent sequestration into intracellular compartments. The mechanism by which astressin achieves this differs greatly from both the “classic” sequestration that follows stimulation with CRF, and from the mechanism used to endocytose PTH₁ following binding to PTH(7-34), the only other example of a class B receptor antagonist known to induce internalization(23). We show here that the CRF₁ receptor undergoes rapid phosphorylation following CRF binding, probably by GRKs since GRK3 has been implicated in the regulation of these receptors(40,41). Similarly, phosphorylation of PTH₁ is observed when either PTH or PTH(7-34) are bound, although the kinases responsible appear to be GRK2 and PKC(34,42). Astressin binding to CRF₁ receptors, however, does not induce any detectable level of phosphorylation, indicating the receptor does not adopt a conformation that is recognized as a substrate by GRKs. Furthermore, CRF and PTH both induce the recruitment of cytosolic βarrestins to their receptors, while neither PTH(7-34) nor astressin are capable of this(34,42,43). Thus, it appears that both CRF₁ and PTH₁ receptors adopt distinct conformations following binding to their agonists or antagonists: the agonists (CRF and PTH) place both receptors into conformations that are recognized as kinase substrates and as βarrestin binding sites, and presumably become desensitized in the process; PTH(7-34) appears to be capable of placing PTH₁ into a conformation that is phosphorylated but not recognized as a binding partner by βarrestins, while astressin-bound CRF₁ receptor is neither a kinase substrate nor a βarrestin binding partner. This model of multiple receptor conformations possessing distinct signaling and regulatory properties is supported by a number of studies that demonstrate regulation and activation of GPCRs are completely separable events. For example, introduction of a zinc ion bridge between transmembrane helices 3 and 6 of PTH₁ constrains the receptor in a conformation that cannot couple to G proteins in response to agonist, but which still permits its phosphorylation and internalization(42). Furthermore, several GPCRs for which multiple agonists or antagonists have been described undergo distinct signaling and regulatory events in a ligand-specific manner. Examples of these include the AT₁A receptor, for which there exist synthetic angiotensin II analogs capable of promoting both signaling and endocytosis, while other analogs only induce internalization(21,22); the µ opioid receptor, where morphine and etorphine both potently activate signaling, but only etorphine induces receptor internalization(44,45); and the chemokine receptor CCR7, where binding of only one of its two endogenous ligands promotes receptor phosphorylation and internalization, whilst both fully activate signaling(46).

Most cells sequester GPCRs through clathrin-coated pits, although other pathways can also be used, for example, caveolae mediate the internalization of both endothelin A and B receptors and possibly also vasoactive intestinal
peptide receptors(16). Our study shows that CRF-induced receptor internalization is likely a clathrin-mediated process, since it is blocked by known inhibitors of clathrin-coated pit function (hypertonic sucrose solution(27) and potassium ion depletion(28)) and by over-expression of the GTPase-deficient K44A mutant of dynamin1, whose activity is required for detachment of clathrin-coated vesicles from the plasma membrane(47). Whilst hypertonic sucrose and K44A dynamin are not specific inhibitors of clathrin-mediated endocytosis, sensitivity to both these treatments and to potassium depletion indicate that CRF-driven internalization is likely a clathrin-mediated event. In contrast, astressin-mediated internalization is not affected by these treatments, indicating that an entirely different pathway is utilized. Since dynamin activity is also required for caveolae-mediated internalization(35,36), the lack of any effect of over-expression of K44A dynamin suggests that this is not the alternative pathway. We confirmed this by showing that neither the disruption of caveolae with filipin III(29), nor over-expression of the caveolin1 S80E dominant negative mutant has any effect on astressin-induced endocytosis of the receptor(26). This differs from the pattern observed for PTH1, where clathrin-mediated endocytosis is used following both PTH and PTH(7-34) binding(23,34). However, the use of distinct endocytic pathways for the same receptor in response to multiple ligands has been described for other GPCRs, including CXCR3, where two of the three agonists (CXCL9 and CXCL10) induce internalization through a dynamin-dependent mechanism, while the third (CXCL11) does not; the nature of the alternative pathway was not investigated further(48).

In addition to clathrin-coated pits and caveolae, cells utilize several other pathways to endocytose membrane-bound receptors, including the well-described processes of phagocytosis and fluid-phase internalization through pinocytosis, as well as some dynamin-independent mechanisms for which there are as yet no tools available to define their exact nature(49). Furthermore, several of these ill-defined mechanisms have been implicated in the endocytosis of GPCRs, including the bradykinin type 2, N-formyl peptide and M2 muscarinic receptors(16). It is interesting to note that the class B family receptor for secretin also internalizes in a dynamin-independent manner, raising the possibility that the CRF1 receptor internalizes through the same mechanism, and that it may represent a general mechanism for class B receptor internalization(50).

In addition to utilizing different paths of CRF1 receptor internalization, we also demonstrated that astressin and CRF binding determine distinct fates for the receptor, since only CRF-bound receptors were subsequently targeted for downregulation. This process requires the receptors to be internalized first since trapping the receptors on the cell surface with the cross-linking agent concanavalin A prior to treatment with CRF inhibited their downregulation. Thus, astressin and CRF target the receptors to alternative intracellular compartments where they either remain sequestered or are downregulated. Several studies of GPCR trafficking have identified interactions between the extreme carboxyl terminal tails of GPCRs with PDZ domain-containing proteins (named after the first three proteins in which they were characterized: PSD-95/ Dlg and ZO-1) as critical for determining receptor fate following internalization(17,30,51,52). The tails of both CRF1 and CRF2 receptors contain putative PDZ-binding motifs and therefore might also be regulated by PDZ proteins. It is interesting to note that an interaction between PTH1 and the PDZ protein Na+/H+ exchanger regulatory factor 2 (NHERF2) has been shown to inhibit PTH(7-34) internalization in descending convoluted tube cells(23). Deletion of the PDZ-binding motif from PTH1 relieves this inhibition and allows PTH(7-34) to induce a level of receptor internalization equivalent to that observed with PTH. While we cannot discount that such a mechanism of regulation exists for CRF1 receptors, preliminary experiments in which we truncated the C-terminal tail, in effect ablating the PDZ-binding motif, did not enhance astressin-induced internalization in either CHO-K1 or HEK-293 cells expressing the CRF1 receptor (data not shown). However, since the identities of PDZ proteins that bind CRF1 receptors are currently unknown, their effects on internalization or trafficking cannot be tested directly.

Finally, we have also investigated the basis of the interaction between astressin and the CRF1 receptor responsible for inducing internalization. The existing explanation of how
the CRF\textsubscript{1} receptor binds to peptide agonists is described by a two-domain model in which the amino terminus of the receptor binds with high affinity to the carboxyl terminal portion of the agonist, substantially increasing the local concentration of agonist and so allowing the second weak interaction to occur between the amino terminal region of the peptide and the juxtamembrane domain of the receptor (7,10). The exact site of this second interaction remains controversial, although contacts between peptide ligands and the second and third extracellular loops of both CRF\textsubscript{1} and CRF\textsubscript{2} receptors have been reported (53-56). Previous data suggest astressin binding is stabilized by an interaction with the juxtamembrane domain of the CRF\textsubscript{1} receptor. In addition, analysis of chimeric receptors suggests strong binding of antagonists to the juxtamembrane domain of CRF\textsubscript{2} receptors (25). In this study we confirmed that astressin binds to the full-length CRF\textsubscript{1} protein with 6-fold higher affinity than to the CRF\textsubscript{1}-ECD/activin IIB receptor construct (Table 1). In contrast, two high affinity 12-residue carboxyl terminal astressin analogs (the Yamada peptides #19 and #20) bind with similar affinities to both proteins. We propose that this difference in the affinities observed for astressin is the result of an additional interaction between the juxtamembrane domain of the receptor and the extra 18 amino acids present in astressin that are absent in the Yamada peptides. Furthermore, this putative interaction appears necessary to induce the conformational change in the receptor required for its internalization, since the short Yamada analogs neither make this contact nor induce internalization.

In summary, we have demonstrated that the CRF\textsubscript{1} receptor is subjected to ligand-specific modes of internalization and trafficking following binding of peptide agonists and antagonists. Furthermore, we present evidence that in order for antagonist binding to promote internalization it must contact both the amino terminus and juxtamembrane domain of the receptor. These findings may have important consequences for the design of CRF\textsubscript{1} receptor antagonists for the treatment of anxiety disorders and depression, since they show that the receptor adopts a distinct conformation when bound to antagonists, which could be exploited to further suppress receptor signaling by inducing its internalization.

REFERENCES

CRF₁ Receptor Internalization By Antagonists


FOOTNOTES

The authors wish to thank Nick Ling for synthesis and purification of peptides, and Khamkeo Khongsaly, Tiffany M. Flynn and Shelby L. Reijmers for additional technical support.

Abbreviations used are: CRF, corticotropin releasing factor; ACTH, adrenocorticotropic hormone; Nle, norleucine; HEK, human embryonic kidney; CHO, Chinese hamster ovary; ECD, extracellular domain; GPCR, G protein-coupled receptor; UCN, urocortin; PTH, parathyroid hormone; GRK, GPCR kinase; 5HT, 5-hydroxytryptamine; GnRH, gonadotropin releasing hormone; AT, angiotensin; FBS, fetal bovine serum; GFP, green fluorescent protein; HEPES, N-[2-hydroxyethyl]piperazine-N’-[2-ethanesulfonic acid]; EDTA, ethylenediaminetetraacetic acid; DMEM, Dulbecco’s modified Eagle’s medium; PBS, phosphate-buffered saline; ELISA, enzyme linked immunosorbent assay; BCA, bicinchoninic acid; PKC, protein kinase C; CCR, C-C chemokine receptor; PDZ, PSD-95/Dlg/ZO-1; NHERF, Na⁺/H⁺ exchanger regulatory factor; CXCL, C-X-C chemokine ligand; GTP₇S, guanosine 5’-O-(3-thiotriphosphate).

FIGURE LEGENDS

Fig. 1. Measurement of agonist and antagonist-induced internalization of CRF₁. A. Time dependent internalization of CRF₁ receptors. CHO-K1 cells expressing FLAG-hCRF₁ receptors were stimulated with 100nM CRF (■) or 100nM astressin (○) or vehicle (×) for between 30 minutes and 24 hours and the amount of receptor remaining at the cell surface was detected using anti-FLAG M2 and anti-mouse-AlexaFluor™488-conjugated antibodies, and measured by flow cytometry. Graph shows mean ± s.e.m. values for % receptor remaining at cell surface normalized to time-matched vehicle-treated controls of 6 independent experiments. B, Dose-dependent internalization of CRF₁ receptors measured after 24 hours stimulation with CRF (■) or astressin (○). Graph shows mean ± s.e.m. values for % receptor remaining at cell surface compared to non-stimulated cells (NS) of 10-14 independent experiments.

Fig. 2. Detection of CRF₁ receptor internalization in mouse corticotrope AtT20 cells. A. Cell surface FLAG-tagged hCRF₁ receptors transiently expressed in AtT20 cells was stained with anti-FLAG M1 antibody conjugated to AlexaFluor™488 and images of representative cells from 3 separate experiments were collected by fluorescence microscopy following 2 hours treatment with vehicle (NT; Ai), 10µM CRF (Aii) or 10µM astressin (Aiii). B, Quantification of FLAG-CRF₁ receptor internalization in AtT20 cells by flow cytometry following stimulation with 10µM CRF (■) or 10µM astressin (○). Values are means ± s.e.m. of 5 or 6 experiments; *, p<0.05, **, p<0.01 compared to time=0.
Fig. 3. Comparison of CRF₁ receptor regulation and internalization by CRF and astressin. A, CHO-K1 cells and CHO-CRF₁ cells were metabolically labeled with ³²P, for 1 hour before stimulation with 100nM astressin or CRF for various times (t). Receptor molecules were immunoprecipitated from lysates made from equal numbers of cells, separated by gel electrophoresis and radiolabeled receptors were quantified and visualized by phosphoimaging. Autoradiograph shown is representative of 3 independent experiments. B, HEK-293 cells were co-transfected with FLAG-CRF₁ receptor and βarrestin2-RmGFP and stained with M1 anti-FLAG antibody-Cy3 conjugate before stimulation with 100nM astressin or CRF for 5 or 15 minutes. Distributions of receptor (red) and βarrestin2-RmGFP (green), and overlays (yellow) were visualized by fluorescence microscopy. C-E, CHO-CRF₁ cells pretreated with medium containing 0.5M sucrose (C), or subjected to K⁺ ion depletion with or without replacement of K⁺ (D), or treated with filipin III (E), were stimulated with 100nM astressin or CRF for 1 hour, and internalized receptor measured by flow cytometry. F, HEK-293 cells co-transfected with FLAG-CRF₁ receptor, empty vector (V), dynamin1 K44A, caveolin1 S80E or caveolin1 S80A were treated with 100nM astressin or CRF for 2 hours and internalized receptor measured by flow cytometry. Data are means ±s.e.m. of 4-6 experiments. Insets are representative western blots demonstrating expression of mutants of dynamin1 and caveolin1 in cell lysates.

Fig. 4. Determination of CRF₁ receptor downregulation following treatment with CRF and astressin. A, CHO-CRF₁ cells were treated with 100nM CRF (■) or astressin (○) for between 1 and 24 hours, and the amount of receptor protein remaining in cell lysates was detected by ELISA (see methods). Data are mean values ± s.e.m. from 3 experiments. The equivalent time course of CRF-induced internalization is included from Fig.1A for comparison (□). B, CHO-CRF₁ cells were treated with 0.25mg/ml concanavalin A prior to stimulation for 24 hours with 100nM astressin or CRF, and internalization and downregulation of the receptor was measured. Data are mean values ± s.e.m. from 4 experiments.

Fig. 5. Comparison of CRF₁ receptor internalization by CRF, astressin and short astressin analogs. A, Binding affinities of astressin ($K_i=0.38nM$) and two 12-residue peptides (Yamada#19, $K_i=1.3nM$ and Yamada#20, $K_i=1nM$) were measured in competition binding assays against $[^{125}I]$-sauvagine on membranes prepared from Ltk⁻ cells expressing the hCRF₁ receptor. Graph shows a representative set of data from one of 3 independent experiments. B, Dose-response curves for internalization of CRF₁ receptors in CHO-CRF₁ cells treated for 16 hours with CRF, astressin, Yamada#19 or Yamada#20. Values are means ± s.e.m. from 4 to 7 experiments.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>$pK_i (K_i, nM)$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$rCRF_1$</td>
</tr>
<tr>
<td>astressin</td>
<td>9.85 ± 0.06ᵃ</td>
</tr>
<tr>
<td>Yamada #19</td>
<td>8.66 ± 0.08ᵇ</td>
</tr>
<tr>
<td>Yamada #20</td>
<td>8.73 ± 0.03ᶜ</td>
</tr>
</tbody>
</table>
Table 1. Inhibition of $[^{125}\text{I}]$-astressin binding to various CRF receptors by astressin analogues. Astressin analogue binding was measured by displacement of $[^{125}\text{I}]$astressin binding to HEK-293 cell membranes in the presence of 30µM GTPγS, for the following receptors: rCRF₁, rCRF₁-ECD (ECD of rCRF₁ receptor attached to the transmembrane domain of the activin IIB receptor), and hCRF₁ receptors. Displacement data were fitted to a single-site competition equation to determine $K_i$, using a $K_d$ value for $[^{125}\text{I}]$astressin of 26pM, 220pM and 65pM for rCRF₁, rCRF₁-ECD and hCRF₁ receptors, respectively. Data are mean ± s.e.m. (n=3-9). Statistical significance of the difference of p$K_i$ value between rCRF₁-NT and rCRF₁ was tested by two-tailed Student’s $t$-test: a – p<0.001; b – p=0.0069; c – p<0.001.
Fig 1

A

CRF$_1$ at cell surface (% time-matched control)

Stimulation Time (h)

B

% CRF$_1$ at cell surface (% of NS)

log[peptide] (M)
Fig2

A

i NT

ii CRF

iii Astr

B

% CRF₁ internalized

0 10 20 30 40 50

0 1 2 3 4

Stimulation time (h)

CRF Astr

* *
Fig 3

A

<table>
<thead>
<tr>
<th>No Receptor</th>
<th>100 nM Astressin</th>
<th>100 nM CRF</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>10</td>
<td>20</td>
</tr>
<tr>
<td>30</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>60</td>
<td>5</td>
<td>20</td>
</tr>
<tr>
<td>t (min)</td>
<td>30</td>
<td>60</td>
</tr>
</tbody>
</table>

B

<table>
<thead>
<tr>
<th>NT</th>
<th>CRF₁</th>
<th>βarr2</th>
<th>overlay</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 min CRF</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>15 min CRF</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 min Astr</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>15 min Astr</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

C

% CRF₁ internalized

D

% CRF₁ internalized

E

% CRF₁ internalized

F

% CRF₁ internalized
Fig4

A

- CRF downreg.
- Astressin downreg.
- CRF internalization

B

- NT
- Con A

% CRF internalized or downregulated

Astellin  CRF  Down-regulation

p<0.05  p<0.005  p<0.01
Distinct conformations of the corticotropin releasing factor type 1 receptor adopted following agonist and antagonist binding are differentially regulated
Stephen J. Perry, Sachiko Junger, Trudy A. Kohout, Sam R. J. Hoare, R. Scott Struthers, Dimitri E. Grigoriadis and Richard A. Maki

J. Biol. Chem. published online January 14, 2005

Access the most updated version of this article at doi: 10.1074/jbc.M412914200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at
http://www.jbc.org/content/early/2005/01/14/jbc.M412914200.citation.full.html#ref-list-1