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Desensitization of human $CRF_{2(a)}$ receptor signaling governed by agonist potency and βarrestin2 recruitment

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ABSTRACT

The primary goal was to determine agonist-specific regulation of $CRF_{2(a)}$ receptor function. Exposure of human retinoblastoma Y79 cells to selective (UCN2, UCN3 or stresscopins) and non-selective (UCN1 or sauvagine) agonists prominently desensitized $CRF_{2(a)}$ receptors in a rapid, concentration-dependent manner. A considerably slower rate and smaller magnitude of desensitization developed in response to the weak agonist CRF. CRF_1 receptor desensitization stimulated by CRF, cortagine or stressin1-A had no effect on $CRF_{2(a)}$ receptor cyclic AMP signaling. Conversely, desensitization of $CRF_{2(a)}$ receptors by UCN2 or UCN3 did not cross-desensitize Gs-coupled CRF_1 receptor signaling. In transfected HEK293 cells, activation of $CRF_{2(a)}$ receptors by UCN2, UCN3 or CRF resulted in receptor phosphorylation and internalization proportional to agonist potency. Neither protein kinase A nor casein kinases mediated $CRF_{2(a)}$ receptor phosphorylation or desensitization. Exposure of HEK293 or U2OS cells to UCN2 or UCN3 (100 nM) produced strong βarrestin2 translocation and colocalization with membrane $CRF_{2(a)}$ receptors while CRF (1 μM) generated only weak βarrestin2 recruitment. βarrestin2 did not internalize with the receptor, however, indicating that transient $CRF_{2(a)}$ receptor–arrestin complexes dissociate at or near the cell membrane. Since deletion of the βarrestin2 gene upregulated Gs-coupled $CRF_{2(a)}$ receptor signaling in MEF cells, a βarrestin2 mechanism restrains Gs-coupled $CRF_{2(a)}$ receptor signaling activated by urocortins. We further conclude that the rate and extent of homologous $CRF_{2(a)}$ receptor desensitization are governed by agonist-specific mechanisms affecting GRK phosphorylation, βarrestin2 recruitment, and internalization thereby producing unique signal transduction profiles that differentially affect the stress response.

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1. Introduction

Hypothalamic-pituitary-adrenal (HPA) axis, defensive behavior, autonomic, metabolic, immune, and cardiovascular responses during stress and trauma are coordinated by the interplay of neuronal corticotropin releasing factor (CRF) and urocortin peptides (UCN1, UCN2, UCN3) differentially binding to and activating CRF receptors type 1 (CRF_1) and type 2 (CRF_2), which are members of the class B1 group of the G protein-coupled receptor (GPCR) superfamily [1–7]. Both CRF receptors are capable of signaling via the protein kinase A (PKA), protein kinase C (PKC), extracellular signal-regulated kinase (ERK) mitogen-activated protein (MAP) kinase, protein kinase B (Akt), and other pathways, although the dominant mode of signal transduction is coupling to G protein subunit Gsα and activating adenylyl cyclase to generate adenosine 3′,5′-cyclic monophosphate (cyclic AMP) [1–7]. CRF_1 receptor signaling generates critical defensive behaviors, HPA hormone secretion, and physiological responses required to survive trauma and stress [1–12]. Behavioral actions mediated by the CRF_2 receptor are complex and contingent upon the brain site and activating agonist unlike the CRF_1 receptor [1,6–9]. Emerging evidence indicates, however, that forebrain CRF_2 receptor signaling can be anxiogenic depending on the intensity and duration

Abbreviations: CK1, casein kinase 1; CK2, casein kinase 2; CRF_1 receptor, CRF receptor type 1; $CRF_{2(a)}$ receptor, CRF receptor type 2(a); K44A, dynamin dominant negative mutant; GPCR, G protein-coupled receptor; GRK, GPCR kinase; PKA, protein kinase A; PKC, protein kinase C; SCP, stresscopin; SRP, stresscopin-related peptide.

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of the stress state [1,9–11]. These CRF receptor-mediated processes must be rapidly initiated in order to meet physiological demands essential for survival. Counter-regulation to restore homeostasis is equally important, however, to prevent stress-induced psychiatric and medical illnesses developing from the detrimental effects of abnormal CRF receptor signaling.

Transduction of cellular signals by G protein-coupled receptors (GPCRs) is stringently regulated to prevent the deleterious effects of unrestrained GPCR signaling. The rapid termination of signaling mediated by agonist-occupied GPCRs is referred to as homologous desensitization and involves the following: (*i*) Agonist-activated receptors selectively recruit a G protein-coupled receptor kinase (GRK) that phosphorylates particular serines and/or threonines in the receptor's intracellular loops or carboxyl-terminus (C-terminus). (*ii*) Phosphorylated receptors attract cytoplasmic βarrestins to the cell membrane where they bind to receptors, uncoupling them from their cognate Gα via competition and steric hindrance. (*iii*) βarrestins subsequently target the receptors to clathrin-coated pits for endocytosis by interacting with clathrin and clathrin adapter protein AP-2. Internalized receptors are either sorted for dephosphorylation and recycling back to the plasma membrane or trafficked into lysosomes for degradation. [13–15]. The stability of the receptor–βarrestin interaction distinguishes two classes of GPCRs, termed class A and class B [15]. Class A GPCRs form transient complexes with βarrestin that dissociate at or near the plasma membrane after the receptors are directed to clathrin coated pits and consequently internalize without βarrestin. Class B receptors form stable complexes with βarrestins that internalize as a unit into endocytic vesicles and persist inside the cell. The stability of the receptor–βarrestin interaction appears to regulate the rate receptors resensitize as well as the spatial-temporal pattern of βarrestin-dependent signaling pathways [14,15].

Recently, βarrestins have been shown to contribute to desensitization and internalization of the CRF_1 and $CRF_{2(b)}$ receptors, the peripherally expressed splice variant of the CRF_2 receptor [16]. There is no information, however, about the homologous regulation of the centrally expressed $CRF_{2(a)}$ receptor by phosphorylation, βarrestin recruitment, and internalization mechanisms. Since abnormal signaling by both CRF receptors in the extended amygdala and forebrain may contribute to the pathophysiology of human stress, anxiety, and depressive disorders, understanding control of CRF_2 receptor function could provide important insight into the pathogenesis of posttraumatic stress disorder and affective illnesses.

Thus, the primary goal of this study was to investigate the desensitization profile of $CRF_{2(a)}$ receptors following exposure to a variety of physiological ligands. We looked not only at the desensitization of cyclic AMP signaling by the $CRF_{2(a)}$ receptor but also at the three primary components of the desensitization process: (*i*) agonist-stimulated phosphorylation, (*ii*) βarrestin recruitment, and (*iii*) receptor internalization. In addition, because we have recently shown that Y79 cells co-express both CRF_1 and $CRF_{2(a)}$ receptors [17], we were able to study potential regulatory interactions between CRF_1 and $CRF_{2(a)}$ receptors in an endogenous setting. Results from our study reveal that desensitization of Gs-coupled $CRF_{2(a)}$ receptor signaling is unaffected by CRF_1 receptor activity and the serine–threonine kinases, protein kinase A and casein kinases, but strongly dependent on agonist potency and βarrestin2 recruitment.

2. Methods and materials

2.1. Reagents

Reagents for cell biology experiments were obtained from the following sources: (1) bovine serum albumin (BSA, fraction V), isobutylmethylxanthine (IBMX), and other highly purified chemicals from Sigma (St. Louis, MO); (2) aprotinin (Trasylol) from Calbiochem (San Diego, CA); (3) forskolin, PKA activators (dbcAMP, SpcAMP-S), PKA inhibitors (H89, RpcAMP-S), and casein kinase inhibitors (TBB, quinalizarin, IC261) from Calbiochem (San Diego, CA); (4) defined fetal bovine serum (#SH30070.03) from Hyclone (Logan, UT). The UCSD Cell Culture Core Facility supplied all other cell culture reagents (Mediatech-CELLGRO). The following CRF receptor agonists were purchased from Bachem (Torrance, CA; purity > 98%) or Phoenix (Belmont, CA; purity >98%) to stimulate cyclic AMP accumulation, and/or to desensitize CRF_1 or CRF_2 receptors: ovine CRF (oCRF); sauvagine (SVG); human/rat CRF (h/rCRF); human urocortin 1 (UCN1), urocortin 2 (UCN2), and urocortin 3 (UCN3); and human stresscopin (SCP) and stresscopin-related peptide (SRP). Drs. Aaron Hsueh and Teddy Hsu (Stanford University) kindly provided stresscopin peptides for the initial experiments. All SDS-PAGE reagents were purchased from Invitrogen-NOVEX (Carlsbad, CA). For CRF_1 receptor phosphorylation experiments, the following reagents were used: (1) protein A sepharose (PrA-Seph) from Oncogene Research Products (Cambridge, MA); (2) the mouse monoclonal anti-HA (HA.11) antibody for immunoprecipitation, the FITC-labeled HA.11 antibody (#FITC-101L) for flow cytometry in transfected HEK293 cells from BAbCO/Covance (Emeryville, CA); (3) the Alexa Fluor 488-labeled HA.11 antibody (#A488-101L) for flow cytometry in transfected MEF cells from BAbCO/Covance (Emeryville, CA).

2.2. Plasmid cDNAs

The human $CRF_{2(a)}$ receptor was previously amplified from a human retinoblastoma Y79 cell complementary DNA (cDNA) library by PCR and subcloned into pcDNA3 (Invitrogen, San Diego, CA) using *Kpn*I and *Xba*I sites [18]. The influenza hemagglutinin (HA) epitope tag (YPYDVPDYA) was inserted between residues Ala^{17} and Glu^{18}, which is an inert region of the amino-terminus (N-terminus) [19], using oligo-directed mutagenesis (Quick Change™ kit, Stratagene, La Jolla, CA). Cyclic AMP signaling by HA-tagged and wild-type $CRF_{2(a)}$ receptor were similar (data not shown). Construction of the βarrestin2–green fluorescent protein (GFP) expression vector has been described previously [15]. Sequences of cDNA constructs were confirmed using single-stranded DNA sequencing.

2.3. Cell culture and transfection

Suspension human retinoblastoma Y79 cultures were grown at a density of 2×10^7 cells/flask in RPMI-1640 and used between passages 4–25 as previously described [17,20]. Human embryonic kidney HEK293 cells were transfected with the cDNA encoding the HA-tagged human $CRF_{2(a)}$ receptor as previously reported [18]. Cyclic AMP signaling experiments confirmed that the sensitivity (i.e., half-maximal effective concentration, EC_{50}) and maximum for agonist-stimulated cyclic AMP accumulation were not altered by insertion of the HA epitope tag in the $CRF_{2(a)}$ receptor's N-terminus (data not shown). Likewise, the binding affinity of the $CRF_{2(a)}$ receptor for its agonists was also not altered by tagging the $CRF_{2(a)}$ receptor with the HA epitope (data not shown). For phosphorylation experiments, transiently transfected HEK293 cells were seeded at 6×10^5 cells/10-cm dish in DMEM containing 10% (v/v) FBS, 100 μg/mL streptomycin, 100 IU/mL penicillin. For confocal microscopy, HEK293 cells were cultured and transiently transfected as previously described [21]. For quantification of βarrestin–GFP translocation, human osteosarcoma U2OS cells (American Type Culture Collection) were cultured in MEM supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum, 10 mM HEPES, 2 mM L-glutamine, and 10 μg/mL gentamicin [21]. For other experiments, wild-type (expressing both βarrestins) and βarrestin2 knockout mouse embryonic fibroblast (MEF) cell lines derived from wild-type and βarrestin2 knockout mice were cultured as previously described [22], and transiently transfected with human $CRF_{2(a)}$ receptor pcDNA3 using a Amaxa Biosystems Nucleofector II and following established Amaxa

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