CRF$_1$ Receptor Signaling via the ERK1/2-MAP and Akt Kinase Cascades: Roles of Src, EGF Receptor, and PI3-Kinase Mechanisms

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In the present study, we determined the cellular regulators of ERK1/2 and Akt signaling pathways in response to human CRF$_1$ receptor (CRF$_1$R) activation in transfected COS-7 cells. We found that Pertussis Toxin (PTX) treatment or sequestering G$_{\beta\gamma}$ reduced CRF$_1$R-mediated activation of ERK1/2, suggesting the involvement of a G$_i$-linked cascade. Neither G$_s$/PKA nor G$_q$/PKC were associated with ERK1/2 activation. Besides, CRF induced EGF receptor (EGFR) phosphorylation at Tyr$^{1068}$, and selective inhibition of EGFR kinase activity by AG1478 strongly inhibited the CRF$_1$R-mediated phosphorylation of ERK1/2, indicating the participation of EGFR transactivation. Furthermore, CRF-induced ERK1/2 phosphorylation was not altered by pretreatment with batimastat, GM6001, or an HB-EGF antibody indicating that metalloproteinase processing of HB-EGF ligands is not required for the CRF-mediated EGFR transactivation. We also observed that CRF induced Src and PYK2 phosphorylation in a G$_{\beta\gamma}$-dependent manner. Additionally, using the specific Src kinase inhibitor PP2 and the dominant-negative-SrcYF-KM, it was revealed that CRF-stimulated ERK1/2 phosphorylation depends on Src activation. PP2 also blocked the effect of CRF on Src and EGFR (Tyr$^{845}$) phosphorylation, further demonstrating the centrality of Src. We identified the formation of a protein complex consisting of CRF$_1$R, Src, and EGFR facilitates EGFR transactivation and CRF$_1$R ERK signaling. PI3K inhibitors were able to inhibit not only the CRF-induced phosphorylation of Akt, as expected, but also ERK1/2 activation by CRF suggesting a PI3K dependency in the CRF$_1$R ERK signaling. Finally, CRF-stimulated ERK1/2 activation was similar in the wild-type CRF$_1$R and the phosphorylation-deficient CRF$_1$R-$\Delta$386 mutant, which has impaired agonist-dependent $\beta$-arrestin-2 recruitment; however, this situation...
may have resulted from the low β-arrestin expression in the COS-7 cells. When β-arrestin-2 was overexpressed in COS-7 cells, CRF-stimulated ERK1/2 phosphorylation was markedly upregulated. These findings indicate that on the base of a constitutive CRF1R/EGFR interaction, the Gβγ subunits upstream activation of Src, PYK2, PI3K, and transactivation of the EGFR are required for CRF1R signaling via the ERK1/2-MAP kinase pathway. In contrast, Akt activation via CRF1R is mediated by the Src/PI3K pathway with little contribution of EGFR transactivation.

Keywords: corticotropin-releasing factor, CRF1 receptor, EGF receptor transactivation, ERK1/2, Src, PI3K/Akt

INTRODUCTION

Behavioral, cognitive, neuroendocrine, and autonomic responses to stress are regulated by CRF1 and CRF2 receptors (CRF1R and CRF2R) (1–3). The preferred mode of signal transduction by both CRF receptors was initially believed to be activation of the Gs/adenyl cyclase/PKA signaling pathway (1–3). Subsequently, CRF1R and CRF2R were also found to signal via the PLC/PKC cascade stimulating intracellular calcium mobilization and IP3 formation (1–4). Besides, both CRF receptors can activate mitogen-activated protein (MAP) kinase cascades in neuronal, cardiac, and myometrial cells endogenously expressing CRF1R or CRF2R and in recombinant cell lines expressing either receptor (2, 3, 5, 6). Several reports suggested that cellular background directed CRF1R to signal selectively via a specific MAP kinase pathway. For example, agonist-activated CRF1R stimulated phosphorylation of ERK1/2 and p38 MAP kinases in PC12 and fetal microglial cells (7, 8) while CRF2R activated ERK1/2 but not JNK and p38 in CHO cells (9). In human mast cells and HaCaT keratinocytes, on the other hand, CRF1R induce phosphorylation of p38 but not ERK or JNK MAP kinases (10, 11). Most studies suggest, however, that the ERK1/2 cascade is the MAP kinase pathway preferentially used by CRF receptors (5, 9, 12, 13).

Signaling via the cyclic AMP (cAMP)-PKA pathway by Gs-coupled GPCRs has been proposed to mediate upstream activation of the ERK cascade in cells with high B-Raf expression (14). Consistent with this concept, PKA regulates CRF1R-mediated ERK activation and ERK-dependent Elk1 transcription in AtT-20 pituitary cells that express high B-Raf levels (15). Kageyama et al. (16) found, however, that ERK activation by CRF1R was mediated by a PKA-independent mechanism in AtT-20 cells. Moreover, other studies have reported that PKA does not play a role in CRF1R ERK signaling in rat CATH.a and rat fetal microglial cells, locus coeruleus neurons, and transfected CHO cells (8, 9, 12, 17). CRF1R can also activate the ERK1/2 cascade via a PKC-dependent mechanism, based on data showing that pretreatment with a PLC or PKC inhibitor blocked urocortin 1 (Ucn1)-stimulated phosphorylation of ERK1/2 in CRF1R-expressing human myometrial, CHO, and HEK293 cells (12, 13), and in rat hippocampal neurons (18). PKC inhibitor pretreatment, however, failed to block CRF- and Ucn1-stimulated ERK1/2 phosphorylation in CRF1R-expressing pituitary AtT20 cells and brain-derived CATH.a cells expressing both CRF receptors (12, 16). These findings suggest that cellular background may also govern the ability of PKA or PKC pathways to regulate CRF1R ERK1/2 signaling similar to its possible role in mediating CRF1R selective activation of a specific MAP kinase cascade.

MEK1/2-mediated phosphorylation of ERK1/2 at Thr202 and Tyr204 during CRF1R and CRF2R signaling in various cell lines has been confirmed by inhibiting ERK1/2 activation with PD98059 (2, 9, 12, 13, 19). Inhibiting C-Raf function by pretreatment with R1-K1 inhibitor or blocking Ras activation by transfection with the dominant-negative mutant RasS17N inhibited Ucn1-stimulated ERK1/2 phosphorylation in CRF1R-expressing CHO and HEK293 cells (5, 12). CRF1R activation by urocortin 2 (Ucn2) and urocortin 3 (Ucn3) has also been shown to signal via the Ras→C-Raf→MEK1/2 cascade in rat cardiomyocytes, based on the ability of manumycin A (a Ras inhibitor) and R1-K1 to abolish ERK1/2 phosphorylation (19). Other research has provided evidence for a phosphoinositide 3-kinase (PI3K)-dependent mechanism contributing to CRF1R- and CRF2R-mediated ERK1/2 activation in HEK293, CHO, A7r5, and CATH.a cells (5, 9, 12). EGFR receptor (EGFR) transactivation, possibly by matrix metalloproteinase (MMP)-mediated ligand release, has been shown to contribute to Ucn1-stimulated ERK1/2 phosphorylation in HEK293 cells, although the mechanisms for CRF1R-mediated transactivation of the EGFR were not determined (5). Furthermore, another study reported that CRF receptor ERK signaling in the mouse atrial HL-1 cardiomyocyte line involved activation of Src (20).

In addition, activation of CRF1R or CRF2(b)R can stimulate phosphorylation of Akt (5, 21). CRF2(b)R Akt signaling in HEK293 cells is mediated by pertussis-sensitive G proteins and PI3K but not by cAMP-stimulated activation of PKA or EPAC, or by PKC (21). The mechanisms regulating Akt signal transduction by CRF1R, however, have not been investigated. Because upstream kinase pathway mediation of CRF1R signal transduction via the ERK and Akt cascades are not well-understood, the primary goal of this study was to test the hypothesis that Src tyrosine kinase and EGFR transactivation

Abbreviations: CRF, Corticotropin-releasing factor; CRF1R, corticotropin-releasing factor receptor type 1; ct-β-AR, β-adrenergic receptor kinase carboxyl terminus peptide; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; ERK1/2, extracellular regulated kinases 1 and 2; GPCR, G protein-coupled receptor; MMP, matrix metalloproteinase; MAP kinase, mitogen-activated protein kinase; PYK2, proline-rich tyrosine kinase 2; PI3K, phosphatidylinositol 3-kinase; c-Src, human homolog of the v-Src Rous sarcoma proto-oncogene; Ucn, urocortin.
are essential regulators of these CRF₁R signaling pathways. We also sought to determine the relative importance of G protein βγ subunits, second messenger kinases, and PI3K in the activation of the ERK1/2 and Akt cascades by the CRF₁R. The results of our study indicate that upstream utilization of Src and PI3K are involved in ERK and Akt signal transduction by the agonist-activated CRF₁R in COS-7 cells, without mediation by PKA and PKC, while transactivation of the EGFR is mainly required for CRF₁R to stimulate phosphorylation of ERK but not for Akt activation.

**MATERIALS AND METHODS**

**Materials**

General reagents utilized were as follows: (i) DMEM, fetal bovine serum (FBS), antibiotic solutions and other cell culture reagents from Invitrogen/GIBCO (Carlsbad, CA); (ii) Pertussis Toxin, reagents for electrophoresis and other highly pure chemicals from Sigma-Aldrich (St. Louis, MO). Human/rat CRF was purchased from Bachem (Torrance, CA). Phorbol 12-myristate 13-acetate (PMA), and the following specific inhibitors were purchased from Calbiochem (La Jolla, CA): Src inhibitor PP2; EGFR tyrosine kinase inhibitor AG1478; PKA inhibitor H89; PKC inhibitor bisindolylmaleimide I, BIM; PI3K inhibitorswortmannin and LY294002; MMP inhibitor GM6001, and Protease Inhibitor Cocktail Set III. BB-94 (batimastat) was obtained from British Biotechnology Ltd (Oxford, UK). Antibodies for Western blots were obtained from the following sources: (i) phospho-p44/42 MAP kinase (Thr²⁰²/Tyr²⁰⁴), total ERK1/2, total EGFR and phospho-c-Src (Tyr⁴¹⁶) from Cell Signaling Technology (Beverly, MA); (ii) total ERK2, total c-Src and total Akt from Santa Cruz Biotechnology (Santa Cruz, CA); (iii) phospho-EGFR (Tyr¹⁰⁶³), phospho-EGFR (Tyr¹¹⁷³) and phospho-EGFR (Tyr¹⁷²) from Biosource-Invitrogen (Carlsbad, CA); (iv) phospho-Akt (Ser⁴⁷³) from Biosource International (Camarillo, CA); (v) phospho-PYK2 (Tyr⁴⁰²) from Calbiochem (La Jolla, CA); (vi) polyclonal anti-human HB-EGF antibody from R&D Systems (Minneapolis, MN); (vii) secondary antibodies conjugated to horseradish peroxidase from Zymed Laboratories (San Francisco, CA).

**DNA Constructs**

The HA-epitope tagging human CRF₁R (HA-CRF₁R), the HA-CRF₁R Δ386 mutant, and the β-arrestin-2 constructs were previously described (22–24). Plasmid pRK5 encoding the carboxyl terminus of βARK that contains its βγ-binding domain (ct-βARK) was kindly provided by Dr. W. Koch (Center for Translational Medicine, Temple University, Philadelphia, PA), which is a scavenger for G protein βγ subunits (25). The expression vector pCEFL-SrcYF-KM, which contains the inactive form of SrcYF, SrcYF-KM (dominant-negative, dn-Src), was kindly provided by Dr. Silvio Gutkind (Department of Pharmacology, UCSD, La Jolla, CA) (26). Plasmid pUSEamp encoding dominant-negative Akt-K179M (dn-Akt) was from Upstate Biotechnology (Lake Placid, NY).

**Cell Culture and Transfection**

COS-7 cells (from the American Type Culture Collection) were cultured at 37°C in a humidified atmosphere of 95% air, 5% CO₂, in DMEM supplemented with 10% FBS, 100 µg/ml streptomycin, and 100 units/ml penicillin (COS-7 growth medium). Transient transfections were performed using LipofectAMINE (Life Technologies: Gaithersburg, MD) as described previously (27). Cells were seeded at 8 × 10⁵ cells/10-cm dish in COS-7 medium and cultured for 3 days before transfection. COS-7 cells were transfected in 5 ml/dish OptiMEM containing 10 µg/ml LipofectAMINE with empty vector, pcDNA3 encoding the HA-CRF₁R or the HA-CRF₁R–Δ386 mutant. In certain experiments, cells were co-transfected with plasmids containing: HA-CRF₁R and mock (empty vector); HA-CRF₁R and ct-βARK; HA-CRF₁R and dn-Src; HA-CRF₁R and dn-Akt, or HA-CRF₁R and full-length β-arrestin-2. After replacing the transfection medium with fresh growth medium, transfected COS-7 cells were cultured for 1 day. Subsequently, cells were re-seeded in 6-well plates and cultured for an additional day prior to the experiment.

**Western Blot Methods**

The protocols for measuring total and phosphorylated ERK1/2, c-Src, PYK2, Akt, and EGFR have been previously published (28, 29). After cells were cultured to 60–70% confluence, they were serum-deprived for 24 h. On the day of the experiment, cells were treated with the indicated ligands and inhibitors. No significant changes in the basal level of ERK1/2 or Akt phosphorylation were observed in cells pretreated with inhibitors, except for BIM, which showed a small increase in ERK1/2 activation (Supplementary Figure S1). After treatment, cells were placed on ice, the media was aspirated, and the cells were washed twice with ice-cold PBS and lysed in 100 µl of Laemmli sample buffer 1X. The lysates were briefly sonicated, heated at 95°C for 5 min, and centrifuged for 5 min at 14,000 rpm. Resulting supernatants were loaded in separate lanes of a 10% SDS-PAGE gels and electrophoresed. Next, Western transfer on to PVDF membranes was completed. The Western blots were then probed with specific antibodies targeting phosphorylated and non-phosphorylated forms of ERK1/2, c-Src, PYK2, Akt, and EGFR for primary immunodetection. After blots were probed with horseradish peroxidase-conjugated secondary antibody, protein bands were visualized with enhanced chemiluminescence ECL reagent (Amersham Pharmacia Biotech, Piscataway, NJ or Pierce Biotechnology, Rockford, IL) and scanned using the GS-800 Calibrated Imaging Densitometer (Bio-Rad). The labeled bands were quantified using the Quantity One 4.6.3 software program (Bio-Rad).

**Co-immunoprecipitation Assay**

COS-7 cells transfected with HA-CRF₁R were grown in 10-cm dishes and serum-deprived for 24 h before treatment with 100 nM CRF for 10 min at 37°C. Cells were washed twice with ice-cold PBS and lysed in Nonidet P-40 solubilization buffer (50 mM Tris-HCl, 150 mM NaCl, 2 mM Orthovanadate, 1 mM NaF, 1% Nonidet P-40, 10% Glycerol, 2 mM EDTA, pH 7.4, containing protease inhibitors). After immunoprecipitation of HA-CRF₁R with anti-HA monoclonal antibody (HA.11;
Covance, San Diego, CA) and protein A/G PLUS-Agarose (Santa Cruz Biotechnology, CA), the proteins were resolved by SDS-PAGE, Western blotted, and probed with anti-EGFR polyclonal or anti-HA monoclonal antibodies, followed by a horseradish peroxidase conjugate to identify co-immunoprecipitated proteins. Blots were also stripped with stripping buffer (100 nM Glycine–HCl, pH 2.7) and reprobed with anti-c-Src polyclonal antibody. Western blot detection of co-immunoprecipitated Src was carried out as described above. Blots were visualized and quantified, as indicated above.

**Statistical Analysis**

Data are presented as mean ± S.E.M. Analyses of variances (ANOVA)s across experimental groups were performed using PRISMTM, Version 8.0 for macOS (GraphPad Software, Inc., San Diego, CA). If the one-way ANOVA was statistically significant, planned post-hoc analyses were performed using Dunnet or Bonferroni’s multiple comparison tests to determine individual group differences.

**RESULTS**

**CRF-Induced ERK1/2 Phosphorylation Is Dependent on Gq Protein and the Gβγ Subunits**

CRF treatment (100 nM) of COS-7 cells transiently transfected with HA-CRF1R caused transient phosphorylation of ERK1/2 that reached a peak at 5–10 min and declined thereafter toward the basal level over the next 30 min (Figure 1A). CRF (100 nM) also caused time-dependent phosphorylation of ERK1/2 in CRF1R-expressing HEK293 and CHO-K1 cells (data not shown), but the rate and magnitude of CRF-induced ERK1/2 activation was considerably less in these cell lines compared to COS-7 cells. In contrast, CRF1Rs expressed in SK-N-MC neuroblastoma cells (4) failed to signal via the ERK1/2 cascade while fibroblast growth factor induced strong ERK1/2 phosphorylation in this cell line (Supplementary Figure S2). Therefore, all subsequent experiments studying ERK1/2 signaling were performed in COS-7 cells transfected with HA-CRF1R cDNA. CRF-induced ERK1/2 activation was concentration-dependent, with a significant increase at 10 nM CRF (~2.4-fold increase over control) and maximal effect over the 0.1–1 μM range (~5.2-fold increase over control, Figure 1B). The EC50 was 25 nM and the maximum occurred at 100 nM for the CRF-induced ERK1/2 phosphorylation.

Most of the known actions of CRF1Rs are mediated through the Gq/PKA signaling cascade, but some of the physiological actions of CRF are also known to occur through activation of Gi or Gβγ proteins (30). To determine the contributions of Gq/PKA-dependent mechanisms to MAP kinase activation, COS-7 cells were pretreated with the PKA inhibitor H89 (500 nM) for 30 min prior to stimulation with CRF (100 nM). As shown in Figure 2A, the PKA inhibitor failed to inhibit CRF-stimulated ERK1/2 phosphorylation. Furthermore, the magnitude of CRF1R-mediated activation of ERK1/2 was similar in COS-7 cells pretreated for 30 min with the highly selective PKA inhibitor Rp-cAMP (0–100 μM) or vehicle (Supplementary Figure S3). We next explored the involvement of Gi/PKC in CRF1R ERK signaling. A 30-min pretreatment of COS-7 cells with the PKC inhibitor BIM (1 μM), increased (~1.6-fold increase over CRF stimulation) rather than decreased CRF-stimulated ERK1/2 activation (Figure 2B). In contrast, BIM pretreatment inhibited ERK1/2 phosphorylation resulting from PMA-induced PKC activation (Figure 2B). Thus, our data suggest that neither Gi/PKA nor Gβγ/PKC are required for the CRF1R-mediated ERK1/2 signaling in COS-7 cells.

On the other hand, the release of Gβγ subunits during GPCR coupling to G protein, particularly through Gq, has an important role in downstream signaling in the ERK1/2 cascade (31, 32). Thus, we examined the role of Gq and Gβγ in CRF-stimulated ERK1/2 activation by two different experimental approaches: treatment with the Gi protein inhibitor pertussis toxin (PTX) and by co-transfecting COS-7 cells with plasmids encoding the carboxyl terminus of βARK containing its βγ-binding domain (ct-βARK) (Supplementary Figure S4), which sequesters βγ, and the CRF1R. COS-7 cells expressing CRF1Rs pretreated with 100 ng/ml PTX showed a marked reduction in CRF-induced ERK1/2 phosphorylation (Figure 2C), suggesting the coupling of CRF1R to Gi to mediate ERK activation. Moreover, overexpressing ct-βARK in COS-7 cells co-expressing CRF1Rs significantly reduced CRF-induced ERK1/2 phosphorylation by ~40% (Figure 2D). Thus, our data implicate Gβγ subunits from PTX-sensitive heterotrimeric G proteins in CRF1R-mediated activation of ERK1/2.

**Transactivation of the EGFR During CRF1R ERK1/2 Signaling**

Because transactivation of receptor tyrosine kinases (RTKs), especially the EGFR, is often an important mechanism used by GPCRs to activate ERK1/2 (33, 34), we investigated the role of EGFR transactivation in CRF1R-mediated ERK signaling. In COS-7 cells transiently transfected with HA-CRF1R, EGF stimulation of the endogenous EGFRs caused ERK1/2 phosphorylation in a time-dependent manner, reaching a maximum effect after 5 min of stimulation, which persisted for at least 30 min (~6.0-fold increase over time 0, Figure 3A). ERK1/2 phosphorylation was also increased by EGF (0–100 ng/ml) in a concentration-dependent manner (EC50 = 0.23 ng/ml, Figure 3B). Thus, these results are consistent with the well-established role of EGFR in ERK1/2 signaling (35).

When COS-7 cells expressing HA-CRF1R were pretreated with the EGFR tyrosine kinase inhibitor AG1478 (100 nM, 30 min), a significant inhibition (~80%) of CRF-induced maximal ERK1/2 phosphorylation was observed (Figure 3C). A concentration-dependent inhibition was observed with AG1478 concentrations of 0–1,000 nM with an IC50 of 10 nM (Figure 3D). Importantly, phosphorylation of the EGFR at Tyr1068 was detected with Western blots in COS-7 cells beginning at 2 min and becoming maximal at 5–10 min of CRF exposure (100 nM) (Figure 3E). Tyr1173 of the EGFR was phosphorylated...
in parallel with Tyr<sup>1068</sup> in COS-7 cells stimulated with CRF (Supplementary Figure S5). Together, these results indicate that CRF-activated CRF<sub>1</sub>R triggers phosphorylation of two critical amino acids located within the autophosphorylation loop that are required for EGFR activation (36, 37). Thus, CRF<sub>1</sub>R signaling rapidly transactivates the EGFR, in agreement with a study reporting that Ucn1 stimulated EGFR transactivation in CRF<sub>1</sub>R-expressing HEK293 cells (5).

MMPs catalyze the release of extracellular heparin-binding EGF (HB-EGF) ligand, which, in turn, binds to and activates the EGFR, thereby stimulating ERK1/2 phosphorylation (38–40). Although this process represents a significant mechanism for GPCR-mediated EGFR transactivation, we found that basal and CRF-stimulated ERK1/2 phosphorylation in transfected COS-7 cells was not altered by inhibiting the formation of the ligand HB-EGF with broad-spectrum MMP inhibitors batimastat BB-94 (5 µM) (Figure 4A) or GM6001 (0–20 µM) (Figure 4B). Similarly, blocking HB-EGF binding to the EGFR with an HB-EGF antibody (5 µg/ml) also failed to inhibit CRF-stimulated ERK1/2 phosphorylation (Figure 4C). In agreement with previous reports (39, 41), GM6001 pretreatment significantly attenuated ERK1/2 phosphorylation induced by PMA but not EGF (Supplementary Figure S6). Altogether, these results exclude a role of MMP in CRF-induced transactivation of the EGFR and subsequent phosphorylation of ERK1/2 in COS-7 cells.

**Src Mediation of CRF<sub>1</sub>R ERK1/2 Signaling**

We then investigated the role of Src kinase, which can serve as an important upstream regulator of GPCR signaling via the ERK1/2 cascade (42, 43). Importantly, 100 nM CRF caused marked phosphorylation of Src at Tyr<sup>416</sup>, which is a requirement for Src activation (44), reaching a maximum at 10 min (~3.5-fold increase over time 0), and persisting for more than 30 min (Figure 5A). This activation was dependent on G<sub>βγ</sub> release since ct-βARK expression reduced the CRF-induced Src phosphorylation (Figure 5B), and as expected, CRF-induced Src phosphorylation was prevented by pretreatment with the selective Src family kinase inhibitor PP2 (Figure 9B). To further evaluate the role of Src in CRF<sub>1</sub>R ERK1/2 signaling, COS-7 cells were co-transfected with the CRF<sub>1</sub>R and a dn-Src. Overexpression of inactive Src prevented ERK1/2 activation by CRF (Figure 5C). Other experiments demonstrated that PP2 pretreatment abolished CRF-stimulated ERK1/2 phosphorylation (Figure 5D), in a concentration-dependent manner (0–20 µM, IC<sub>50</sub> = 2 µM) (Figure 5E). These findings support our hypothesis that Src plays a central role in CRF<sub>1</sub>R ERK1/2 signaling.

We next determined if CRF-stimulated Src activation is required for CRF<sub>1</sub>R-induced transactivation of EGFRs. In this context, previous research has established that Src can activate EGFR signaling by phosphorylating Tyr<sup>845</sup> of the EGFR protein (45, 46). As shown in Figure 6A, we found that 100 nM CRF stimulated in a time-dependent manner marked phosphorylation
of EGFR at Tyr\textsuperscript{845} beginning at 2 min and becoming maximal at 10 min. This effect was blocked by pretreatment of the cells with PP2 (Figure 6B). In a recent study by Perkovska et al. (47), it was shown that V\textsubscript{1b} vasopressin receptor interacts with Src at basal state, suggesting the formation of a GPCR/Src complex that facilitates MAP kinase activation. To evaluate if a CRF\textsubscript{1}R/Src complex exists under basal conditions, we analyzed CRF\textsubscript{1}R immunoprecipitates for the presence of co-precipitated Src under basal and CRF-stimulated conditions. As shown in Figure 6C, 100 nM CRF induced a robust interaction between the CRF\textsubscript{1}R and Src after 10 min stimulation (\sim 8.0-fold increase over control). Interestingly, it was also observed that under the same immunoprecipitation conditions, the EGFR is also present in the CRF\textsubscript{1}R/co-precipitated complex, even in the absence of CRF stimulation (Figure 6D). After stimulation with 100 nM CRF for 10 min, we observed a significant increase in the CRF\textsubscript{1}R/EGFR interaction (\sim 2.5-fold increase over control). These observations suggest that CRF promotes the formation of a multiprotein complex that would allow rapid EGFR phosphorylation at Tyr\textsuperscript{845} by Src, present in this complex.

It has been shown that, in parallel to Src activation by many GPCRs, the proline-rich tyrosine kinase 2, PYK2, is also phosphorylated and activated, and in association with
FIGURE 3 | Involvement of EGFR transactivation in CRF-stimulated ERK1/2 phosphorylation. COS-7 cells expressing HA-CRF₁Rs were treated with 10 ng/ml EGF for the indicated times (A) or exposed to the indicated concentration (B) of EGF for 10 min. COS-7 cells expressing HA-CRF₁Rs were pretreated with 100 nM (C) or (Continued)
Src is required for the subsequent transactivation of EGFR (44, 48). Therefore, we decided to assess whether activation of CRF$_R$R leads to PYK2 phosphorylation in COS-7 cells. As shown in Figure 6E, 100 nM CRF caused rapid phosphorylation of PYK2 in a time-dependent manner (0–30 min), reaching a maximum effect at 5 min and persisting for at least 30 min of stimulation. Interestingly, and as expected, CRF-mediated PYK2 phosphorylation was also dependent on G$\beta$γ release (Figure 5B).

**PI3K Mediation of CRF$_R$ ERK1/2 and Akt Signaling**

PI3Ks can mediate important biological actions of GPCRs, including cell proliferation or survival, by serving as an upstream regulator of Akt and ERK cascades (49, 50). As shown in Figure 7A, 100 nM CRF caused rapid phosphorylation of Akt, an effect that was decreased by PTX pretreatment (Figure 7B) or ct-βARK overexpression (Figure 7C), similar to the previously observed effect on the CRF-induced ERK1/2 phosphorylation,
FIGURE 5 | Role of Src tyrosine kinase in CRF-stimulated ERK1/2 phosphorylation. (A) COS-7 cells expressing HA-CRF1Rs were stimulated with 100 nM CRF for the indicated times. (B) COS-7 cells co-transfected with a plasmid pRK5 encoding the carboxyl terminus of βARK that contains its βγ-binding domain (ct-βARK) or an empty control vector (Mock) and the pcDNA3-HA-CRF1R expression vector were stimulated with 100 nM CRF for 5 or 10 min. (C) COS-7 cells co-transfected with a dominant-negative SrcYF-KM (dn-Src) expression vector or an empty control vector (Mock) and the pcDNA3-HA-CRF1R expression vector were stimulated with 100 nM CRF for 5, 10, or 15 min.

(Continued)
suggesting the participation of Gι protein and Gβγ subunits in this process. It is important to note that none of the observed effects of PTX and ct-βARK on CRF actions were present on EGF-stimulated ERK1/2 and Akt phosphorylation (Supplementary Figure S7).

Pretreatment with selective PI3K inhibitors, wortmannin (100 nM) (Figure 8A), or LY294002 (10 μM) (Figure 9C) abolished CRF1/R-mediated Akt signaling activation. Similarly, inhibition of PI3K by 100 nM wortmannin abolished the stimulatory action of EGF on Akt (Figure 8A), thereby demonstrating that the PI3K pathway is required for both CRF- and EGF-induced Akt phosphorylation. Considering that an upstream PI3K mechanism can also regulate CRF1/R and CRF1/R signaling via the ERK1/2 cascade in A7r5, CATH.a, and transfected CHO cells (9, 12), we investigated the potential role of PI3K in the activation of ERK1/2 by HA-CRF1Rs expressed in COS-7 cells. In this context, activation of RTKs, such as the EGFRs, has been shown to recruit PI3K and activate ERK1/2 (50–53). However, contradictory data on PI3K involvement in EGFR-induced ERK1/2 phosphorylation have been reported (54–56). In this regard, to find out if EGFR-mediated ERK1/2 phosphorylation observed in COS-7 cells is depending on PI3K activation, we analyze the effect of 100 nM wortmannin on the EGF ERK1/2 activation. As shown in Figure 8A, pretreatment with wortmannin was unable to inhibit the effect of EGF, suggesting that PI3K does not participate in this mechanism. In contrast, pretreatment with wortmannin abolished CRF-stimulated ERK1/2 phosphorylation (Figure 8A) in a concentration-dependent manner (0–100 nM), confirming an intermediary role for PI3K in CRF1/R ERK signaling (Figure 8B). To examine the contribution of CRF-mediated activation of Akt to the phosphorylation of ERK1/2, we evaluated the effect of the dn-Akt mutant. As shown in Figure 8C, overexpression of dn-Akt had no significant effect on ERK1/2 activation after stimulation with CRF (Figure 8C), suggesting that Akt does not participate in the activation of ERK1/2 by CRF. Because in the present work we do not show evidence of impairment of kinase activity of the dn-Akt, it will be necessary the use of other approaches, such as genetic tools or inhibitors, to provide more evidence regarding the possible no effect of Akt on the ERK 1/2 pathway. Because in the present work we do not show evidence about impairment of kinase activity of the dn-Akt, it will be necessary the use of other approaches, such as genetic tools or inhibitors, to provide more evidence regarding the possible Akt lack of effect on ERK 1/2 pathway. Consequently, our results suggest that PI3K can regulate the transduction of CRF1/R signals through the ERK cascade, possibly independently of Akt.

Src Acts Upstream and PI3K Downstream of the EGFR During CRF-Induced ERK1/2 Activation

Since we found that CRF-induced EGFR transactivation mediates ERK1/2 phosphorylation through Src- and PI3K-dependent mechanisms, we next determined if CRF-induced PI3K activation occurs upstream or downstream of Src and EGFR. Wortmannin inhibition of PI3K had no effect on CRF-induced phosphorylation of EGFR at Tyr845 (Figure 9A), suggesting that PI3K acts downstream of Src and EGFR. Consistent with these results, we also observed that wortmannin pretreatment did not alter CRF-induced phosphorylation of Src at Tyr416 (Figure 9B). Therefore, CRF1/R-stimulated transactivation of EGFR and phosphorylation of ERK1/2 mediated by Src was independent of PI3K. It has been reported that the PI3K/Akt signaling pathway can be activated at least by two independent mechanisms: (i) EGFR transactivation (57), and (ii) upstream Src activation (58, 59). We observed that CRF-induced Akt activation was completely inhibited by the Src inhibitor, PP2 (Figure 9C), suggesting that Src is an upstream regulator of PI3K and Akt. We next measured the effect of the specific EGFR tyrosine kinase inhibitor, tyrphostin AG1478, on CRF-stimulated Akt phosphorylation. As observed in Figure 9D, while CRF-induced ERK1/2 activation was totally dependent on EGFR transactivation (Figure 9D), Akt phosphorylation was only partially dependent. Thus, we hypothesize that PI3K/Akt pathway signaling by CRF1/R may involve two mechanisms: (i) a strong dependence on upstream Src activating PI3K and then Akt (Figure 9C); (ii) a weak dependence on EGFR transactivation (Figure 9D).

Role of β-Arrestin-2 in the CRF-Mediated ERK1/2 Activation

In recent years, it has been identified that β-arrestin proteins play an important role in mediating the actions of GPCRs, particularly those related to activation and regulation of Src and mitogenic pathways, in particular, the ERK1/2 signaling cascade (60). To determine the role of β-arrestins in the CRF-mediated ERK1/2 activation observed above, we used a phosphorylation-deficient mutant CRF1/R, which also shows a diminished agonist-dependent β-arrestin-2 recruitment (24). As shown in Figure 10A, COS-7 cells transiently transfected with HA-CRF1/R-Δ386 mutant showed a similar response in ERK1/2 phosphorylation compared to that observed with CRF1/R. The apparent independence of CRF-mediated activation of ERK1/2 from β-arrestin-2 could be explained by the low β-arrestin expression level.
CRF mediates EGFR phosphorylation at Tyr845 and activation of PYK2. COS-7 cells expressing HA-CRF Rs were stimulated with 100 nM CRF for the indicated times (A,E) or pretreated with 10 µM PP2 for 30 min before stimulation with 100 nM CRF for 5 min (B). Total cell lysates were separated by SDS-PAGE (Continued).
previously detected in COS-7 cells (61, 62). To assess this possibility, we evaluated the effect of β-arrestin-2 overexpression in COS-7 cells, since CRF₁R activation has been shown to lead to selective recruitment of β-arrestin-2 in both HEK293 cells and neurons (24, 63). As observed in Figure 10B, cells co-expressing HA-CRF₁R and β-arrestin-2 showed a significant increase in the CRF-mediated ERK1/2 phosphorylation, suggesting that β-arrestin involvement in CRF₁R ERK1/2 signaling depends on its cellular expression levels.

**DISCUSSION**

In the present study, we investigated the molecular mechanisms associated with the activation of ERK1/2 and Akt signaling cascades by the human CRF₁R in COS-7 cells. Our data suggest that agonist-stimulated CRF₁R promotes Gᵢ activation and Gβγ release which, in turn, stimulate phosphorylation and activation of Src kinase. Once Src is active, it mediates ERK1/2 phosphorylation by at least two independent signaling mechanisms: (i) phosphorylation and transactivation of the EGFR, (ii) activation of PI3K. Interestingly, CRF₁R-induced Akt phosphorylation also requires Src-mediated activation of PI3K as the main mechanism, but it is mostly independent of EGFR transactivation.

Defining the molecular mechanisms for ERK1/2 signaling by a GPCR has become a significant focus of signal transduction research due to the multifaceted pathways mediating signaling via the ERK1/2-MAP kinase cascade. A significant role of the ERK1/2-MAP kinase pathway has been recognized in the biological action of both CRF₁R and CRF₂R. ERK1/2 is widely distributed in the brain and is considered an essential regulator of the molecular processes involved in response to stress (6, 64). It is well-established that most GPCRs signal via ERK1/2-MAP kinase cascades through distinct Gᵢ, Gₛ, and Gβγ-dependent signaling pathways. In the case of the CRF₁R, it has been identified that the Gₛ/PKA pathway is importantly involved in the activation of MAP kinase cascades (12, 15, 18). In contrast, we found that pretreating CRF₁R-expressing COS-7 cells with PKA inhibitors H89 or Rp-cAMP did not alter the ability of CRF to stimulate ERK1/2 phosphorylation. Although earlier research proposed that high cellular expression of the serine-threonine kinase B-Raf molecularly switches “upstream” ERK1/2 activation by Gₛ-coupled GPCRs to a PKA mechanism (14), pretreating fetal hippocampal cells with the PKA inhibitor H89 only produced a small reduction in CRF₁R-mediated ERK phosphorylation despite very high hippocampal levels of B-Raf (18). Furthermore, H89 failed to inhibit CRF₁R-mediated ERK signaling in brain-derived CATH.a, rat fetal microglial, locus coeruleus, and transfected CHO cells (8, 9, 12, 17). In fact, ERK activation by CRF₁R in HEK293 cells was markedly decreased after the third intracellular loop’s Ser⁷⁰⁰ was phosphorylated by PKA (65). Thus, a cAMP-dependent PKA → Rap1 → B-Raf mechanism does not always mediate ERK1/2 signaling by Gₛ-coupled receptors. EPAC, a guanine nucleotide exchange factor that is activated by intracellular cAMP, has been shown to regulate activation of Rap1 and ERK1/2 without the involvement of PKA (66). Gₛ-coupled CRF₁R signaling can stimulate ERK1/2 phosphorylation by activating upstream EPAC2 independent of PKA in certain cell lines (17, 67). Interestingly, neither Epac nor PKA was found to mediate Akt cascade signaling by CRF₂R in HEK293 (21).

The versatility of the CRF₁R to activate different signaling pathways has allowed its coupling to Gₛ proteins to be identified (4). Gₛ conveys a signal to activate PKC which then triggers MAP kinase cascades. Thus, it has been shown that Gₛ/PLC/PKC cascade signaling by CRF₁R activated by Ucn1 contributes to phosphorylation of ERK1/2 in CRF₁R-expressing myometrial, CHO, HEK293, and rat hippocampal cells (12, 13, 18). However, in pituitary AtT20 cells and CATH.a cells, PKC is not involved in Ucn1-stimulated ERK1/2 phosphorylation (12). In our study, pretreatment with the PKC inhibitor, BIM, increased rather than reduced CRF-stimulated ERK1/2 phosphorylation, suggesting that PKC may negatively regulate CRF₁R ERK1/2 signaling in COS-7 cells, although the specific mechanism for this effect remains to be determined.

The use of PTX in our study suggests the participation of Gᵢ protein in the CRF-dependent activation of ERK and Akt pathways. Interestingly, it is now well-established that during GPCR/Gᵢ signaling, Gβγ release can activate a myriad of effectors to modulate diverse signaling pathways downstream of GPCRs, including Src, which in turn activate EGFR to promote ERK1/2 activation (43, 68, 69). Gβγ-activated Src can also associate PYK2. When we blocked that action of Gβγ subunits in COS-7 cells by overexpressing the ct-βARK peptide, which is a Gβγ subunit scavenger (70, 71), CRF-stimulated ERK phosphorylation was decreased by ~40%. Moreover, ct-βARK overexpression markedly reduced phosphorylation of Src and Akt. In agreement, another group has also found that CRF₁R ERK1/2 signaling is only partially dependent on Gβγ, although their study did not assess the role of Gβγ subunits in the activation of upstream ERK1/2 pathways.

Differences in CRF₁R-mediated activation of the ERK1/2-MAP kinase cascade are probably attributable to variations...
FIGURE 7 | CRF mediates Akt activation. (A) COS-7 cells expressing HA-CRF₁Rs were stimulated with 100 nM CRF for the indicated times. (B) COS-7 cells expressing HA-CRF₁Rs were pretreated with 100 ng/ml PTX for 15 h before stimulation with 100 nM CRF for 5 min. (C) COS-7 cells co-transfected with a plasmid pRK5 encoding the carboxyl terminus of βARK that contains its γβ-binding domain (ct-βARK) or an empty control vector (Mock) and the pcDNA3-HA-CRF₁R expression vector were stimulated with 100 nM CRF for the indicated times. Total cell lysates were separated by SDS-PAGE and analyzed by immunoblotting with anti-p-Akt Ser⁴⁷³, as described in Materials and Methods. Akt phosphorylation was quantitated by densitometry, and mean values were plotted from three to five independent experiments. Vertical lines represent the S.E.M. Western blots were also probed for total Akt showing equal loading. (A) b,p<0.01 vs. 0 min. (B) d,p<0.001 vs. Con, c,p<0.001 vs. PTX; a,p<0.05 vs. CRF (–). (C) b,p<0.01 vs. CRF₁R/Mock (5 and 10 min).
in the signaling properties of transfected CRF$_1$Rs expressed in different cell lines utilized in these studies. We are presently investigating other upstream factors including β-arrestins that regulate Src and EGFR mediation of CRF$_1$R ERK1/2 signaling.

Our experiments did demonstrate that CRF-stimulated phosphorylation of ERK1/2 and EGFR occurred in parallel, while pretreatment with the EGFR kinase inhibitor, AG1478, caused a concentration-dependent inhibition of CRF-stimulated ERK1/2 phosphorylation. In agreement, it has been shown that EGFR transactivation is required for Ucn1-stimulated ERK1/2 phosphorylation in transfected HEK293 cells (5). In contrast to our data indicating that a MMP/HB-EGF ligand mechanism was not involved, however, this group reported that MMP generation of an HB-EGF ligand transactivated the EGFR during CRF$_1$R ERK1/2 signaling (5). Therefore, EGFR transactivation can play a critical role in CRF$_1$R signaling via the ERK1/2-MAP kinase cascade.
Earlier studies have implicated a PI3K-dependent mechanism in CRF₁R ERK1/2 signaling based on the observation that pretreatment with PI3K inhibitors attenuated sauvaquine- and Ucn1-stimulated ERK1/2 phosphorylation in CRF₁R-expressing CHO and HEK293 cells (5, 9, 12). PI3K is also involved in CRF₂B₃R-stimulated ERK1/2 activation in CHO, A7r5, and mouse neonatal cardiomyocyte cells (12, 19). However, the activation sequence of PI3K, EGFR, and ERK1/2 during CRF₁R signaling has not been fully elucidated. Here we observed that pretreating CRF₁R-expressing COS-7 cells with the PI3K inhibitors wortmannin and LY294002 inhibited CRF-stimulated phosphorylation of ERK1/2 and Akt. Previous studies suggest that PI3K activity is required for Gβγ-mediated MAP kinase signaling pathway at a point upstream of Sos and Ras activation (50, 72).
Because we also found that AG1478 abolished phosphorylation of ERK1/2 while only decreasing Akt phosphorylation 25% in transfected COS-7 cells stimulated with CRF, upstream activation of the PI3K/Akt pathway by CRF₁R is not strongly dependent on EGFR transactivation. In this context, our study suggests that Src acts as a critical mediator of PI3K activation, independent of EGFR transactivation, which, in turn, stimulates Akt and ERK1/2 phosphorylation. Previous studies have shown that activated Src directly associates with PI3K through interaction between the SH3 domain of Src and the proline-rich motif in the p85 regulatory subunit of PI3K, thereby increasing the specific activity of PI3K (59). Furthermore, intermediary proteins have also been identified to mediate Src-induced PI3K activation, such as p66Shc, Rap1, and FAK. Thus, our study raises the possibility that Src activates PI3K, although the specific mechanism for this effect remains to be determined.

For certain GPCRs, Src has been shown to induce EGFR transactivation, stimulate the PI3K-Akt pathway, and activate the ERK1/2 cascade (43, 48, 70). A novel finding in our study is the rapid and parallel phosphorylation of Src, PYK2, the EGFR, Akt, and ERK1/2 in CRF₁R-expressing COS-7 cells stimulated with CRF. Importantly, we demonstrated that inhibiting Src function with PP2 markedly reduced or abolished the CRF-stimulated activation of Src, PYK2, the EGFR, and ERK1/2, suggesting that Src has a central role in regulating CRF₁R ERK1/2 signaling. Thus, our results clearly show that Src triggers signal transduction by two important pathways culminating in ERK activation by CRF₁R: (i) EGFR activation of the classical Ras/Raf/MEK/ERK pathway, and (ii) PI3K regulation and subsequent activation of ERK1/2 (Figure 11).

To the best of our knowledge, our study demonstrates for the first time that Src regulates ERK and Akt signaling by the CRF₁R. Yuan et al. (20) reported that Src was an upstream regulator of ERK signaling by both the CRF₁R and CRF₂R in the mouse atrial HL-1 cardiomyocytes cell line based on the effects of antalarmin (a CRF₁R antagonist) and antisauvagine (a CRF₂R antagonist). Although CRF₁R was reported to be expressed in the human heart (73, 74), Ikeda et al. (75) reported that CRF₂(b)R is the major CRF receptor expressed in the HL-1 mouse atrial cardiomyocyte cell line with no measurable level of CRF₁R mRNA. Their data detecting only
CRF₂R expression in HL-1 cells is consistent with previous and more recent studies demonstrating only CRF₂R expression in rat and mouse cardiomyocytes (19, 76, 77). Therefore, ERK1/2 signaling stimulated by Ucn in cardiomyocytes is mediated through CRF₁R, which appears to be the main mediator of the cardiac stress response (78, 79), rather than through CRF₂R. Additionally, recent observations also indicate that CRF₂R controls the cellular organization and colon cancer progression, specifically through the Src/ERK pathway (80, 81). Thus, while all previous findings are relevant to CRF₂Rs, our findings show for the first time that Src plays an important role in the regulation of ERK1/2 and Akt signaling by the CRF₁R.

An important finding of our study was the detection of a signaling protein scaffold, which contains CRF₁R, Src, and EGFR (Figures 6C,D). While the association between CRF₁R and Src was totally dependent on CRF agonist activation, a constitutive interaction between CRF₁R and EGFR was also detected, which was increased after CRF stimulation. In this context, it has previously reported that some GPCRs physically interact with EGFR in the absence of receptor ligands, a condition that may increase the efficiency of EGFR transactivation (29, 82–84). Thus, it is possible that the detected constitutive association between CRF₁R and EGFR facilitates a more rapid CRF agonist-induced recruitment of Src to the EGFR and subsequent phosphorylation and activation of EGFR. With regard to this possibility, the presence of a putative proline-rich domain-binding SH3 motif (ProXXPro; X, any amino acid), located in the carboxyl terminus of the CRF₁R (Pro398Thr399Ser400Pro401) may provide a site for the direct interaction between Src and CRF₁R after agonist stimulation. However, the CRF₁R-Δ386 mutant, which lacks the ProXXPro motif, induces a similar degree of ERK1/2 activation that is induced by the wild-type CRF₁R, which suggests this putative region may not participate in the binding to Src (Figure 10A).

Moreover, there is evidence that Tyr phosphorylation of GPCRs plays a role in mediating GPCR-Src interactions (43). For instance, in studies conducted in A431 epidermoid carcinoma cells, stimulation of the β₂-adrenergic receptor (β₂-AR) with isoproterenol, results in phosphorylation of the receptor on Tyr305 (43, 85). The mutation of this residue to Phe abolishes Src/β₂-AR association and impairs Src activation. This residue lies within a canonical Src SH2 binding domain, and it is proposed that Src directly binds the Tyr-phosphorylated β₂-AR. Interestingly, the CRF₁R also has a single putative SH2 binding domain (TyrXX-hyd; hyd, hydrophobic amino acid) located at the end of the third intracellular loop (Tyr309Arg310Lys311Ala312), which may play a role in mediating the interaction between Src and CRF₁R.
which may be a site where Src can directly interact with CRF1_R. Further work is needed to establish the importance of this putative site in the agonist-induced CRF1_R/Src interaction and Src activation.

β-arrestins are a small family of cytosolic proteins initially identified for their central role in GPCRs desensitization. Furthermore, β-arrestins act as adaptors in clathrin-mediated receptor endocytosis (86). In this sense, their role in CRF1_R homologous desensitization and endocytosis is well-recognized, particularly for β-arrestin-2 (24, 63, 87). It is now well-established, however, that β-arrestins can also act as GPCR-signaling transducers that recruit and activate many other signaling molecules, including Src, MAP kinase, NF-κB and PI3K that modulate diverse cellular responses (64, 86). β-arrestin regulation of CRF/CRF1_R signaling is still not fully understood.

Regarding β-arrestin regulation of CRF/CRF1_R-mediated ERK1/2 activation, β-arrestin-2-meditation of CRF1_R internalization participates in the late phase of sustained ERK1/2 activation after G protein activation and B-Raf mediate the early phase of ERK1/2 activation (88). However, overexpression of PDS-95 in HEK293 cells, a CRF1_R-interacting protein, inhibited CRF-induced-CRF1_R internalization in a PDZ-binding motif-dependent manner by suppressing β-arrestin-2 recruitment. Intriguingly, neither the overexpression of PSD-95 nor the knockdown of endogenous PSD-95 affected CRF-mediated activation of ERK1/2 (89).

Under this experimental evidence and due to the importance of β-arrestins in the scaffolding and activation of Src and regulation of MAP kinase cascades, it was decided to evaluate their role in the CRF/CRF1_R-mediated ERK1/2 activation observed in COS-7 cells. Using a phosphorylation-deficient mutant CRF1_R, which has a decreased interaction with β-arrestin-2 (24), no significant changes in the activation of ERK1/2 were detected after agonist stimulation (Figure 10A), suggesting that β-arrestin-2 is not involved in the CRF/CRF1_R-mediated ERK1/2 activation observed in COS-7. This finding, however, can be explained in part by the low expression level of β-arrestins in COS-7 cells (61, 62). This hypothesis is supported by our data showing that overexpressing β-arrestin-2 in COS-7 notably increased the CRF/CRF1_R-mediated ERK1/2 activation (Figure 10B). Likewise, β-arrestin overexpression in COS-7 cells has been found to augment CRF1_R internalization (24). Thus, our data provide evidence about the involvement of β-arrestin-2 in the CRF/CRF1_R MAP kinase activation in cells with sufficient β-arrestin expression.

Our findings on signaling pathways activated by CRF1_R help to elucidate the molecular mechanisms involved in response to stress mediated by this receptor. For instance, kinases in the ERK1/2-MAP kinase cascade, including Src and PYK2, are highly expressed in extended amygdala and forebrain neurons regulating anxiety defensive behavior and stress responsiveness (90–92). Acute stress or central CRF administration induces rapid phosphorylation of ERK1/2 in the basolateral amygdala and hippocampal neurons and prominent anxiety-like behavior in rats and mice (93–95). Furthermore, CRF1_Rs can also signal through other cellular pathways that may be involved in post-traumatic stress disorder pathophysiology. As we showed here, CRF1_R activated by CRF stimulated rapid phosphorylation of Akt at Ser473 that is mediated by upstream Src and PI3K. Preclinical research has shown that activated Akt in the ventral tegmentum promotes resilience to anxiety- and depressive-like responses to stress (3, 96), while high levels of phosphorylated Akt in the dorsal hippocampus and basolateral amygdala prolongs contextual and sensitized fear induced by inescapable stress (3, 97). Therefore, the consequences of CRF1_R Akt signaling during trauma and severe stress may differ depending on the brain region. Hence, ERK1/2-MAP kinase and Akt cascade signaling by CRF1_R regulated by Src, PYK2, and EGFR may have critical roles in stress-induced anxiety and depression.

**CONCLUSIONS**

In summary, the data presented herein establish that the tyrosine kinase Src serves as a central upstream regulator of ERK1/2-MAP kinase and Akt cascade signaling by the human CRF1_R in COS-7 cells. Although CRF1_R coupling to G proteins strongly activates PKA and PKC pathways, neither second messenger kinases were involved in CRF1_R-mediated ERK1/2 signaling. However, Gβγ released during activation of CRF1_R by CRF, particularly from G1i, stimulates phosphorylation of Src and PYK2, which in turn promotes transactivation of the EGFR through the formation of a heterotrimeric complex formed by the association of CRF1_R, Src, and EGFR. EGFR transactivation, which occurred independent of MMP generation of the HB-EGF ligand, was essential for CRF-stimulated ERK1/2 phosphorylation while having only a small role in CRF1_R-mediated Akt activation. Although PI3K activation contributes to CRF-stimulated ERK1/2 phosphorylation, CRF1_R-mediated EGFR transactivation is independent of the PI3K/Akt pathway. In contrast, CRF1_R Akt signaling while also being mediated by generation of Gβγ and phosphorylation of Src is weakly dependent on EGFR transactivation.

**DATA AVAILABILITY STATEMENT**

All datasets generated for this study are included in the article/Supplementary Material.

**AUTHOR CONTRIBUTIONS**

JO-R, RH, and KC conceived the project. JO-R, FD, and RH designed the experiments. GP-M, AF-G, JH-A, and MD-C carried out the experiments. JO-R, GP-M, and AF-G analyzed and discussed the data. JO-R and RH wrote the manuscript. All authors read and approved the final version of the manuscript and took a due care to ensure the integrity of the work.

**FUNDING**

JO-R was supported by the CINVESTAV-IPN, a University of California MEXUS-CONACYT grant for collaborative...
projects, CONACYT grants (48777 and 167673) and a Grant for Research on Health from Fundacion Miguel Aleman-2018. RH was funded by the VA Center of Excellence for Stress and Mental Health (CESMH); a VA Merit Review grant, NIH RO1 grants AG050595 and AG022381 from the National Institute of Aging; and a University of California MEXUS-CONACYT grant for collaborative projects. GP-M held a CONACYT graduated scholarship (296029).

DEDICATION

This work is dedicated to Dr. Kevin J. Catt, who was an extraordinary scientist, mentor, and friend who passed away on October 1, 2017.

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ACKNOWLEDGMENTS

The authors wish to express their gratitude to Dr. Walter Koch for the plasmid pR5K expressing the carboxyl terminus (ßγ-binding domain) of βARK and Silvio Gutkind for the pCFeL-SrcYF-KM which contains the inactive form of SrcYF (dominant-negative). The authors would also like to thank Dr. Jose Antonio Arias-Montaño and M.Sc. Huguet Landa-Galvan, Cinvestav-IPN, Mexico, for their critical comments on the manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2019.00869/full#supplementary-material
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Conflict of Interest: FD was employed by Novaliq GmbH.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be constructed as a potential conflict of interest.

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