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The Relaxin Family Peptide Receptor 3 Activates Extracellular Signal-Regulated Kinase 1/2 through a Protein Kinase C-Dependent Mechanism

Emma T. van der Westhuizen, Tim D. Werry, Patrick M. Sexton, and Roger J. Summers

Department of Pharmacology, Monash University, Clayton, Victoria, Australia

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ABSTRACT

Human gene 3 relaxin (H3 relaxin) is a member of the relaxin/insulin family of peptides. Neuropeptides mediate behavioral responses to stress and regulates appetite; however, the cell signaling mechanisms that control these events remain to be identified. The relaxin family peptide receptor 3 (RXFP3, formerly GPCR135 or SALPR) was characterized as the receptor for H3 relaxin, functionally coupled to the inhibition of cAMP. We have identified that RXFP3 stably expressed in Chinese hamster ovary (CHO)-K1 (CHO-RXFP3) and human embryonic kidney (HEK) 293 (HEK-RXFP3) cells activates extracellular signal-regulated kinase (ERK) 1/2 when stimulated with H3 relaxin and an H3 relaxin B-chain (dimer) peptide. Using inhibitors of cellular signaling proteins, we subsequently determined the mechanism of ERK1/2 activation by RXFP3. ERK1/2 phosphorylation requires the activation of $G_{i/o}$ proteins and seems to require receptor internalization and/or compartmentalization into lipid-rich environments. ERK1/2 activation also predominantly occurred via the activation of a protein kinase C-dependent pathway, although activation of phosphatidylinositol 3-kinase and Src tyrosine kinase were also involved to a lesser extent. The mechanisms underlying ERK1/2 phosphorylation were similar in both CHO-RXFP3 and HEK-RXFP3 cells, although some differences were evident. Phospholipase Cβ and the transactivation of endogenous epidermal growth factor receptors both played a role in RXFP3-mediated ERK1/2 activation in HEK293 cells; however, they were not involved in RXFP3-mediated ERK1/2 activation in the CHO-K1 cell background. The pathways identified in CHO- and HEK-transfected cells were also used in the murine SN56 neuronal cell line, suggesting that these pathways are also important for RXFP3-mediated signaling in the brain.

The relaxin/insulin peptide family includes the relaxins (relaxin-1, relaxin-2, and relaxin-3), insulin, a number of insulin-like peptides (INSL3–6), and the insulin-like growth factors (I and II). These peptide hormones function during pregnancy and in the regulation of cell growth and metabolism (Bathgate et al., 2006a). Relaxin-3 is the most recently identified member of this family of peptides, with the highest mRNA expression in the brain, in the neurons of the nucleus incertus (NI) (Bathgate et al., 2002). In these neurons, relaxin-3 is located in diffusely distributed vesicle-like structures in the cytoplasm and is thus considered a neuromodulator with roles in controlling stress and food intake (McGowan et al., 2005, 2006; Tanaka et al., 2005).

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ABBREVIATIONS: INSL, insulin-like peptide; CHO, Chinese hamster ovary; RXFP3, relaxin family peptide receptor 3; CHO-RXFP3, Chinese hamster ovary-K1 cells stably expressing relaxin family peptide receptor 3; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; H3 relaxin, human gene 3 relaxin; H2 relaxin, human gene 2 relaxin; HEK, human embryonic kidney; HEK-RXFP3, human embryonic kidney 293 cells stably expressing relaxin family peptide receptor 3; NI, nucleus incertus; ERK, extracellular signal-regulated kinase; PI3K, phosphatidylinositol 3-kinase; PDGF, platelet-derived growth factor; PTX, pertussis toxin; SALPR, somatostatin and angiotensin-like peptide receptor; SN56, murine septal neuron-derived cell line; GPCR, G protein-coupled receptor; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase kinase; FBS, fetal bovine serum; PMA, phorbol 12-myristate-13-acetate; PKC, protein kinase C; SH, sulfhydryl; FRT, Flp recombination target; DMEM, Dulbecco's modified Eagle's medium; BSA, bovine serum albumin; ANOVA, analysis of variance; PLC, phospholipase C; Pyr, 2-pyridinesulfenyl; PD98059, 2′-amino-3′-methoxyflavone; U0126, 1,4-diamino-2,3-dicyano-1,4-bis(methylthio)butadiene; LY294002, 2-(4-morpholinyl)-8-phenyl-1(4*H*)-benzopyran-4-one hydrochloride; Gö6976, 12-(2-cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-5*H*-indolo(2,3-*a*)pyrrolo(3,4-*c*)-carbazole; AG1478, 4-(3-chloroanillino)-6,7-dimethoxyquinazoline; U73122, 1-(6-[17β-3-methoxyestra-1,3,5-(10)triene-17-yl]amino/hexyl) 1*H*-pyrroledione; Ro 31-8220, 3-[1-(3-(amidinothio)propyl-1*H*-indol-3-yl)]-3-(1-methyl-1*H*-indol-3-yl) maleimide (bisindolylmaleimide IX); AG370, 2-amino-4(1*H*-indol-5-yl)-1,1,3-tricyanobuta-13-diene.

Stress and anxiety are major contributing factors to depression and can also influence the normal functioning of the nervous, endocrine, and immune systems (Shen et al., 2004; Sasaguri et al., 2005). Stress is characterized by corticotropin-releasing factor-mediated activation of the hypothalamic-pituitary-adrenal axis. The NI neurons are densely populated with corticotropin-releasing factor type 1 receptors (van Pett et al., 2000), which are strongly implicated in processing cortical, cerebellar, and sensory information (Potter et al., 1994). In rats, the forced-swim test increased expression of the immediately early gene c-*Fos* in neurons of the NI, with a subsequent increase in relaxin-3 mRNA, suggesting that relaxin-3 is also important in processing the response to stress (Tanaka et al., 2005). Although strategies currently used to manage stress and depression focus mainly on altering serotonergic and noradrenergic pathways in the brain, the relaxin-3 pathway could provide an alternative therapeutic target.

Human relaxin-3 (H3 relaxin) may also play a role in the control of food intake and obesity. Ingestion is controlled by signals that originate from the digestive tract, after gut distension and cholecystokinin release, that are transmitted to the brain via vagal sensory nerve fibers. The signals are integrated with information from the hypothalamus and other forebrain sites to terminate feeding (Berthoud, 2004). Injection of H3 relaxin into the paraventricular nucleus of the rat brain causes increased food intake in satiated rats (McGowan et al., 2005), suggesting that this peptide may also play a role in the communication of satiety signals to the brain and could thus represent a novel therapeutic target for combating obesity.

The receptor for relaxin-3, RXFP3 (Bathgate et al., 2006a) [formerly GPCR135 (Liu et al., 2003) or SALPR (Matsumoto et al., 2000)] is located in the brain. The receptors are found at the terminal boutons of H3 relaxin-containing projections originating from the cell bodies of the NI, with the highest RXFP3 mRNA levels detected in the paraventricular nucleus of the hypothalamus and the supraoptic nucleus (Liu et al., 2003; Sutton et al., 2004b). RXFP3 is a G protein-coupled receptor (GPCR) that couples to pertussis toxin (PTX)-sensitive G proteins to inhibit forskolin-stimulated cAMP production (Liu et al., 2003). It belongs to a subfamily of GPCRs that includes somatostatin, angiotensin, bradykinin, opioid, and apelin receptors, many of which stimulate the phosphorylation and activation of the mitogen-activated protein kinase (MAPK) extracellular signal-regulated kinase (ERK) 1/2 (Bito et al., 1994; Mannon and Raymond, 1998; Lallemend et al., 2003; Audet et al., 2005; Olivares-Reyes et al., 2005). The pathways coupling these receptors to ERK activation are generally dependent on $G_{i/o}$ or $G_{q/11}$ families of G proteins, but downstream these pathways are highly divergent and somewhat cell type-dependent, indicating receptor- and cell-specific recruitment of intracellular signaling components. RXFP3 coupling to ERK1/2 has not been documented, but because ERK1/2 activation has been implicated in central responses to stress and feeding (Morikawa et al., 2004; Shen et al., 2004; Sutton et al., 2004a; Sasaguri et al., 2005), it may be an effector through which RXFP3 modulates these responses.

We have shown previously in CHO-K1 cells stably expressing RXFP3 that the receptor couples strongly to $G_{i/o}$ proteins, because pretreatment of the cells with pertussis toxin completely abolishes the extracellular acidification response (van der Westhuizen et al., 2005). We also showed that pretreatment with PD98059, an inhibitor of MAPK/ERK kinase (MEK), partially inhibited the acidification response, suggesting that RXFP3 was coupled to ERK. In this study, we show that RXFP3 activates ERK1/2 and have determined the activation mechanism in CHO-K1 (CHO-RXFP3) and HEK293 (HEK-RXFP3) cells stably expressing human RXFP3 receptor and the SN56 neuronal cell line that endogenously expresses mouse RXFP3 receptors.

Materials and Methods

Materials. Flp-In CHO and Flp-In 293 cells, BP clonase and LR clonase, LipofectAMINE, fetal bovine serum (FBS), hygromycin B, pDONR201, pEF5/FRT/V5, and pOG44 vectors were all purchased from Invitrogen (Carlsbad, CA). SN56 cells were a gift from Dr. R. A. D. Bathgate (Howard Florey Institute, Melbourne, Australia). The Ultraclean 15 DNA purification kit was purchased from MoBio-Labs (Solana Beach, CA). EcoRI and XhoI restriction enzymes were purchased from Promega (Madison, WI). Penicillin/streptomycin and amphostat B were from ThermoTRACE (Melbourne, Australia). The cAMP AlphaScreen assay kit, Protein A beads, and white 384-well Optiplates were from PerkinElmer Life and Analytical Sciences (Wellesley, MA). Surefire phospho-ERK kit was from TGR biosciences (Adelaide, Australia). p42/p44 MAP kinase (ERK1/2), phospho-p42/p44 MAP kinase (phospho-ERK1/2), and anti-rabbit IgG-horseradish peroxidase antibodies were from Cell Signaling Technologies (Danvers, MA). PD98059, U0126, LY294002, AG370, chelerythrine chloride, phorbol 12-myristate-13-acetate (PMA), and Gö6976 were purchased from BIOMOL International (Plymouth Meeting, PA). Tyrphostin AG1478, PP1 analog, PP2, Ro 31-8220, ET-18-OCH_3, bisindolylmaleimide I, and protein kinase C (PKC) ζ pseudosubstrate were from Calbiochem (La Jolla, CA). Pertussis toxin (PTX), EGTA, and forskolin were from Sigma Aldrich (St. Louis, MO). ECL enhanced chemiluminescence reagents were from Roche (Mannheim, Germany), and Hyperfilm was from GE Healthcare (Chalfont St. Giles, Buckinghamshire, UK). Tissue culture flasks and plates were from Nunc (Rochester, NY). INSL3, H3 relaxin (Bathgate et al., 2006b), the H3 relaxin B-chain peptides [single B-chain peptides with cysteine 10 protected by either a sulfhydryl (SH) group or 2-pyridinesulfenyl (Pyr) group] (Bathgate et al., 2006b), the H3 relaxin B-chain (dimer) (parallel dimer of two B-chains with one disulfide bond between position 10 cysteines and cysteine 22 attached to acetamidomethyl groups) (Bathgate et al., 2006b), and INSL5 A-chain/relaxin-3 B-chain chimera (Sutton et al., 2004b) were synthesized at the Howard Florey Institute (Victoria, Australia) by Dr. John Wade. INSL5/H3 relaxin chimeric peptide was iodinated by ProSearch (Victoria, Australia) (specific activity, 2200 Ci/mmol). Human relaxin-2 (H2 relaxin) was supplied by BAS Medical (San Mateo, CA), and porcine relaxin was extracted from ovaries and purified at the Howard Florey Institute (Layden and Tregear, 1996).

Receptor Constructs. The cDNA clone for SALPR (now known as RXFP3) was from the UMR cDNA resource center (http://www.cdna.org) (Rolla, MO). RXFP3 sequence was amplified from SALPR-pcDNA3.1+ using the forward primer 5′-GGGACAATTTGTACAAAAAA-CAGGCTTCACCATGCAGATGGCCGATGCAGCC-3′, (start codon underlined) and the reverse primer 5′GGGGACCACTTTGTACAAGAAAGCTGGGTC**CTA**GT-AGGCAGAGCTGCTGG-3′ (stop codon underlined and in boldface type), containing attB1 and attB2 restriction sites at the 5′ and 3′ ends, respectively, using 30 cycles at 94°C for 30 s, 58°C for 30 s, and 72°C for 3 min. The amplified product was purified using the Ultraclean 15 DNA purification kit according to the manufacturer's protocol and cloned into pDONR201 between the attP1 and attP2 restriction sites with BP clonase at 25°C for 16 h. An expression clone of RXFP3 was created with

pEF5/FRT/V5 using LR clonase at 25°C for 3 h. Correct insertion of the RXFP3 sequence into the vectors was confirmed by restriction enzyme digest with EcoRI and XhoI and gel electrophoresis and by BigDye Terminator (v3.1) sequencing (Applied Biosystems, Foster City, CA) performed at the Micromon sequencing facility (Monash University, Victoria, Australia).

Cell Culture. CHO-K1 (Flp-In) and HEK293 (Flp-In) cells are genetically modified to contain a single Flp recombination target (FRT) for recombination with a Flp-In vector. Flp-In CHO and Flp-In 293 cells were cotransfected using 120 ng/1 × 10^6 cells pERF5/FRT/V5-RXFP3 and 1080 ng/1 × 10^6 cells pOG44 (Flp-ase for RXFP3 insertion into the FRT recombinant site) with LipofectAMINE according to the manufacturer's protocol for 5 h at 37°C in a humidified atmosphere. Stable transfectants (CHO-RXFP3 and HEK-RXFP3) were selected by culturing in the presence of 400 ng/ml hygromycin B. CHO-RXFP3 and HEK-RXFP3 cells were maintained in 75-cm^2 flasks at 37°C in a humidified atmosphere with 5% CO_2/95% air in DMEM supplemented with 5% (v/v) FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.5 μg/ml amphostat B, and 400 ng/ml hygromycin B. SN56 cells were also maintained as described above; however, hygromycin B was not included in the growth medium.

Whole-Cell Binding Assays. Cells (1 × 10^5 cells/well) were subcultured into 48-well plates 24 to 48 h before experimentation to achieve ~90% confluence for binding assays and Lowry protein estimation. Binding assays were conducted in a volume of 200 μl per well in binding buffer [DMEM/Ham's F-12 medium + 0.5% bovine serum albumin (BSA)] containing [^{125}I]INSL5/H3 relaxin chimera (25 pM–2 nM) for saturation binding assays or with [^{125}I]INSL5/H3 relaxin chimera (100 pM) for competition binding assays. Cells were incubated with radioligand with gentle shaking for 90 min at 22°C because kinetic experiments demonstrated that binding had equilibrated at this time. Binding buffer was aspirated from the cells, and they were washed twice with ice-cold phosphate-buffered saline and then solubilized in 0.5 M NaOH. Solubilized cells were transferred to scintillation vials, and 3 ml of liquid scintillation cocktail was added per vial, shaken, and incubated overnight in the dark. Vials were counted for 90 s in a liquid scintillation counter (β-counter) (Wallac 1414 WinSpectral; PerkinElmer Life and Analytical Sciences). Nonspecific binding was determined in the presence of 1 μM H3 relaxin.

Inhibition of cAMP Accumulation Assay. Cells (5 × 10^4 cells/well) were subcultured into 96-well plates and grown for 27 h in complete DMEM/Ham's F-12 medium and then serum-starved for 18 h before experimentation. The cAMP AlphaScreen assay kit was used to measure the amount of cAMP generated by the cells upon stimulation with forskolin and its inhibition by H3 relaxin using a modified protocol. In brief, DMEM was removed from the wells and replaced with stimulation buffer [13 mM NaCl, 5.1 mM KCl, 0.77 mM $MgSO_4 \cdot 7H_2O$, 1.23 mM $CaCl_2 \cdot 2H_2O$, 0.32 mM $Na_2HPO_4 \cdot 2H_2O$, 0.42 mM KH_2PO_4, 5.27 mM D-glucose, 0.5 mM 3-isobutyl-1-methylxanthine, 5 mM HEPES, and 0.1% (w/v) BSA, pH 7.4]. Cells were stimulated for 5 min with H3 relaxin (1 pM–1 μM) and then for 3 min with forskolin (30 μM) in a 37°C shaking incubator. Cells were lysed in lysis buffer [0.3% (v/v) Tween 20, 5 mM HEPES, and 0.1% (w/v) BSA, pH 7.4] on ice and frozen at −70°C. Cell lysates were thawed on ice, mixed thoroughly, and a 5-μl sample was transferred to a white 384-well Optiplate. Acceptor beads diluted in detection buffer [5.2 mM NaCl, 2 mM KCl, 0.31 mM $MgSO_4 \cdot 7H_2O$, 0.5 mM $CaCl_2 \cdot 2H_2O$, 0.13 mM $Na_2HPO_4 \cdot 2H_2O$, 0.17 mM KH_2PO_4, 2.1 mM D-glucose, 3 mM HEPES, 1.8% (v/v) Tween 20, and 0.1% (w/v) BSA, pH 7.4] were added to each well under ambient light, and incubated at 22°C in the dark for 30 min. Donor beads and biotinylated cAMP were also diluted in detection buffer and incubated separately at 22°C in the dark for 30 min. Donor bead/biotinylated cAMP mix was added and incubated for 16 h at 22°C in the dark. Plates were read on a Fusion-α microplate plate reader (PerkinElmer Life and Analytical Sciences).

ERK Phosphorylation Assay. Estimation of the level of phosphorylated ERK was performed using the Surefire phospho-ERK kit, a novel non-Western blot-based proprietary technique that relies on the energy transfer-based AlphaScreen (Amplified Luminescent Proximity Homogeneous Assay Screen) concept (Osmond et al., 2005). The cells were plated as for cAMP assay and treated for 2 to 30 min with relaxin peptides at 37°C in the presence or absence of inhibitors. Concentrations of inhibitors used in this study were determined previously (Werry et al., 2005a) by inhibitor concentration-response curves or sourced from the literature. Cell lysates were prepared and processed according to the manufacturer's protocol. In brief, a 5-μl sample of processed cell lysate was transferred to a white 384-well Optiplate, and 6 μl of a 1:1 mix of Protein A AlphaScreen donor and acceptor beads was added to each well. Plates were incubated for 2 h at 22°C in the dark. A Fusion-α microplate reader (PerkinElmer Life and Analytical Sciences) was used to excite the donor beads and to measure the emission of light from the acceptor beads after energy transfer.

Western Blots. CHO-RXFP3 and HEK-RXFP3 cells were plated (1 × 10^6 cells/well) into six-well plates. Cells were stimulated with relaxin peptides for 5 min at 37°C. Media were aspirated, and cells were lysed in 1× SDS sample buffer [62.5 mM Tris-HCl, pH 6.8, 2% (w/v) SDS, 10% (v/v) glycerol, 50 mM dithiothreitol, and 0.01% (w/v) bromphenol blue]. Cells were scraped from the wells and transferred to Microfuge tubes on ice. Samples were sonicated (Hielscher; UP50H) for 1 cycle of 10 s at 100% amplitude and then heated at 95°C for 5 min. Samples were electrophoresed at 100 V for 1 h through 10% SDS-polyacrylamide gels. Protein was transferred to polyvinylidene difluoride membranes using a semidry electroblotter at 1.2 mA × cm^2 for 1 h. Membranes were probed with 1:1000 dilution of p42/p44 MAP kinase (ERK1/2) antibody or phospho-p42/p44 MAP kinase (phospho-ERK1/2) antibody in antibody binding buffer (Tris-buffered saline/Tween 20 and 0.5% BSA) and then a 1:1000 dilution of anti-rabbit IgG-horseradish peroxidase raised in sheep diluted in Tris-buffered saline/Tween 20. Different membranes were used for the determination of phosphorylated ERK1/2 and nonphosphorylated (total) ERK1/2. Bands were visualized using ECL enhanced chemiluminescence reagents and Hyperfilm autoradiography film.

Data Analysis. Data from concentration-response experiments were analyzed using Prism 4.0 (GraphPad Software, Inc., San Diego, CA). Curves were fit using the following three-parameter Hill equation:

$$Y = \text{Basal} + \frac{E_{max} - \text{Basal}}{1 + 10^{(-pEC_{50} - X)}}$$

where X denotes log agonist concentration, Basal denotes the minimal asymptotic response in the absence of agonist, E_{max} denotes the maximum asymptotic response in the presence of agonist, and pEC_{50} denotes the negative logarithm of the concentration of agonist provoking a response halfway between the maximum and minimum asymptotes.

Concentrations and absolute quantities of cAMP were extrapolated from a standard curve (range, 10 pM–1 μM). Data from ERK time course experiments were analyzed with baseline light emission (520–620 nm range) subtracted from all values, and then the results were normalized against the maximal response to H3 relaxin.

Statistical Analysis. Histograms were analyzed using one-way ANOVA with Dunnett's multiple comparison post hoc test. [^{125}I]INSL5/H3 relaxin binding in the absence of peptide (control group) was compared with binding in the presence of relaxin family peptides (treatment groups). ERK1/2 phosphorylation with isoenzyme-specific PKC inhibitors compared the effect of H3 relaxin in the presence of inhibitors to the effect of H3 relaxin alone. Sigmoidal concentration-response curves were analyzed for a one-site versus a two-site fit using PRISM 4.0 (GraphPad Software) and comparing the two models by an F test. H3 relaxin competition binding and inhibition of forskolin-stimulated cAMP accumulation curves were fit to the preferred model, a single-site sigmoidal dose-response

curve. Time course experiments were analyzed using two-way ANOVA, with comparisons made between inhibitor treatment groups and the H3 relaxin control curve at each time point.

Results

Chimeric INSL5/H3 Relaxin Binds to CHO-RXFP3 and HEK-RXFP3 Cells. Binding studies using CHO-RXFP3 and HEK-RXFP3 cells revealed a pharmacological profile similar to that observed in COS-7 cells transiently expressing RXFP3 (Liu et al., 2003). Saturation binding studies were conducted in whole cells with [^{125}I]INSL5/H3 relaxin in CHO-RXFP3 and showed a single population of sites with a density of 24.6 ± 3.10 fmol/mg protein with pK_d = 9.29 ± 0.05 (apparent K_d = 0.51 nM) (n = 5) and in HEK-RXFP3 cells with a single population of sites at a density of 100 ± 4.60 fmol/mg protein with pK_d = 9.21 ± 0.09 (apparent K_d = 0.62 nM) (n = 6). Competition binding studies in whole cells showed in CHO-RXFP3 (Fig. 1A) and HEK-RXFP3 cells (Fig. 1B) that only H3 relaxin and H3 relaxin B-chain (dimer) competed with [^{125}I]INSL5/H3 relaxin for binding. The pIC_{50} values for H3 relaxin were 8.92 ± 0.20 and 9.03 ± 0.20 (n = 4–6) in CHO-RXFP3 (Fig. 1C) and HEK-RXFP3 (Fig. 1D) cells, respectively, and for H3 relaxin B-chain dimer were 6.61 ± 0.45 and 6.96 ± 0.20 (n = 6), respectively (Fig. 1, C and D). No significant competition was observed with H2 relaxin (1 μM), porcine relaxin (1 μM), INSL3 (1 μM), or with H3 relaxin B-chain peptides (1 μM) (Fig. 1, A and B).

RXFP3 Is Functionally Coupled to the Inhibition of cAMP in CHO-RXFP3 and HEK-RXFP3 Cells. RXFP3 has been reported to inhibit adenylate cyclase (Liu et al., 2003). We confirmed this in our RXFP3-expressing CHO and HEK cell lines. Cells were exposed to H3 relaxin (1 pM–0.1 μM; 5 min) followed by forskolin (30 μM; 3 min) and there was a concentration-dependent inhibition of cAMP accumulation (pIC_{50} = 9.17 ± 0.36 in CHO-RXFP3 and 9.82 ± 0.71 in HEK-RXFP3). Inhibition of cAMP production in response to RXFP3 activation was more marked when the receptor was expressed in CHO than in HEK cells (Fig. 2A).

H3 Relaxin and H3 Relaxin B-Chain Dimer Activate ERK1/2. Cells were exposed to H3 relaxin (1 pM to 0.1 μM; 5 min), and there was a concentration-dependent activation of ERK1/2 (pEC_{50} = 9.23 ± 0.24 in CHO-RXFP3 and 9.00 ± 0.08 in HEK-RXFP3) (Fig. 2B). Time course studies were subsequently carried out using H3 relaxin and H3 relaxin B-chain peptides (all 10 nM) and direct measurement of ERK1/2 phosphorylation (pERK1/2). The maximal ERK1/2 activation occurred 2 to 5 min after agonist addition (Fig. 3, A and B). The maximal activation of ERK1/2 was produced by H3 relaxin (10 nM) [maximum response, 6.57 ± 0.78 fold/basal (CHO); 5.81 ± 1.33 fold/basal (HEK)], whereas H3 relaxin B-chain (dimer) (10 nM) produced a smaller response [3.63 ± 1.00 fold/basal (CHO); 3.49 ± 1.46 fold/basal (HEK)]. The H3 relaxin B-chain monomers did not activate ERK1/2 in either cell line (Fig. 3, A and B). No pERK1/2 was detected in untransfected Flp-In CHO or Flp-In 293 cells treated with H3 relaxin, H2 relaxin, porcine relaxin, or INSL3 (all 10 nM; n = 3; data not shown). Western blots confirmed that the pERK1/2 response occurred in CHO-RXFP3 and HEK-RXFP3 cells after 5-min stimulation with H3 relaxin (10 nM), but stimulation with H3 relaxin B-chain (dimer) and the single H3 relaxin B-chain peptides (all 10 nM) produced only a small increase in pERK1/2 (Fig. 3, C and D). ERK1/2

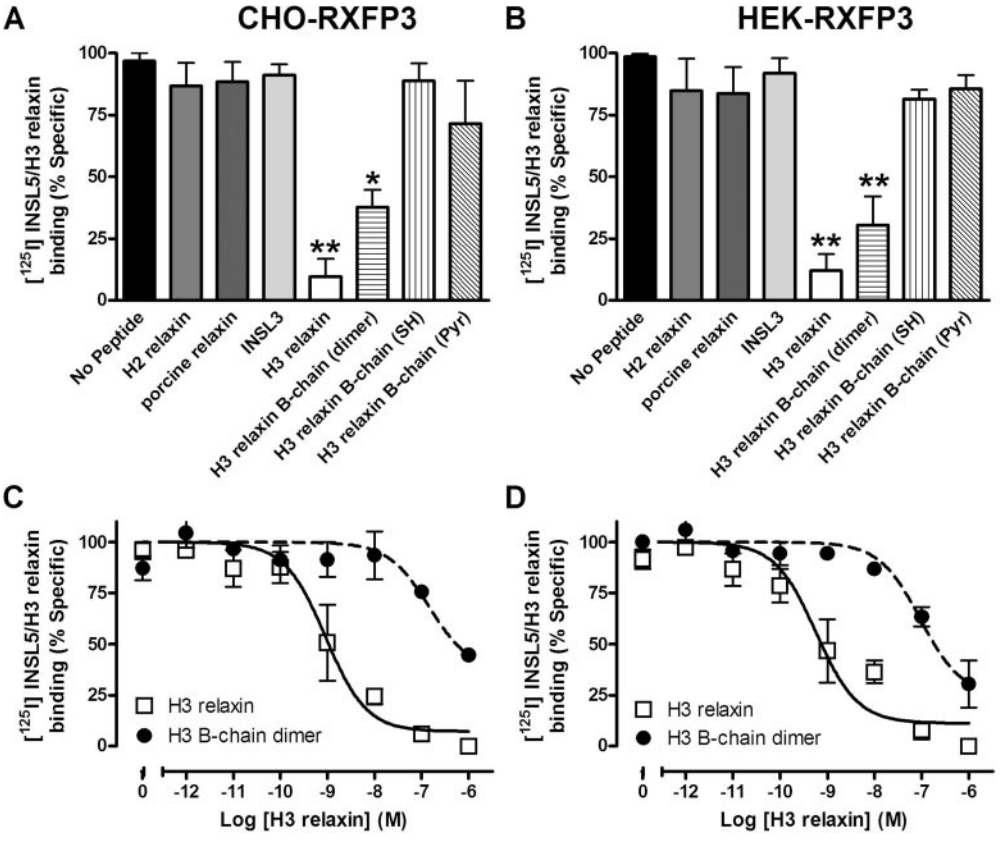

Fig. 1. Competition for [^{125}I] INSL5/H3 relaxin chimera binding (100 pM) at the RXFP3 receptor. Competition binding studies were carried out in CHO-RXFP3 cells (A) and HEK-RXFP3 cells (B). In both cell lines, there was competition for [^{125}I]INSL5/H3 relaxin chimera binding by H3 relaxin (1 μM) and H3 relaxin B-chain (dimer) (1 μM). No competition was observed in the presence of H2 relaxin, porcine relaxin, human INSL3, or two H3 relaxin B-chain peptides [H3 B-chain(SH) and H3 B-chain(Pyr); cysteine 10 protected by either an SH group or Pyr group] (all 1 μM). Competition for [^{125}I]INSL5/H3 relaxin chimera binding by H3 relaxin (C) and H3 relaxin B-chain dimer (D) was concentration-dependent in both cell lines. Results are mean ± S.E.M. for four to six independent experiments performed in triplicate. *, $p < 0.05$, **, $p < 0.01$ compared with [^{125}I]INSL5/H3 relaxin chimera binding alone (one-way ANOVA with Dunnett's multiple comparison post hoc test).

phosphorylation was stronger in response to H3 relaxin stimulation in CHO-RXFP3 cells than HEK-RXFP3 cells, consistent with results obtained with the Surefire ERK kit. No change in pERK1/2 was observed after treatment of FlpIn CHO or FlpIn HEK cells with relaxin family peptides for 5 min (all 10 nM; $n = 3$; data not shown).

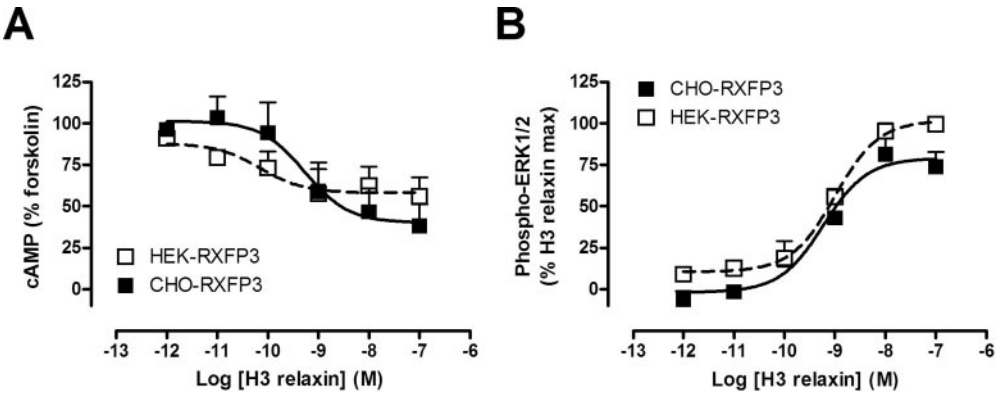

Fig. 2. Inhibition of forskolin-stimulated cAMP accumulation and activation of ERK1/2 by H3 relaxin acting at RXFP3 receptors. Preincubation with H3 relaxin (1 pM to 0.1 μM; 5 min) caused concentration-dependent inhibition of forskolin (30 μM; 3 min)-stimulated cAMP accumulation (A). pIC_{50} values for inhibition of forskolin-stimulated cAMP accumulation were 9.17 ± 0.36 (CHO-RXFP3) and 9.82 ± 0.71 (HEK-RXFP3). Stimulation with H3 relaxin (5 min) caused a concentration-dependent increase in ERK1/2 phosphorylation (B). pEC_{50} values for ERK1/2 activation were 9.23 ± 0.24 (CHO-RXFP3) and 9.00 ± 0.08 (HEK-RXFP3). Data represent mean ± S.E.M. for 6 to 10 independent experiments performed in triplicate.

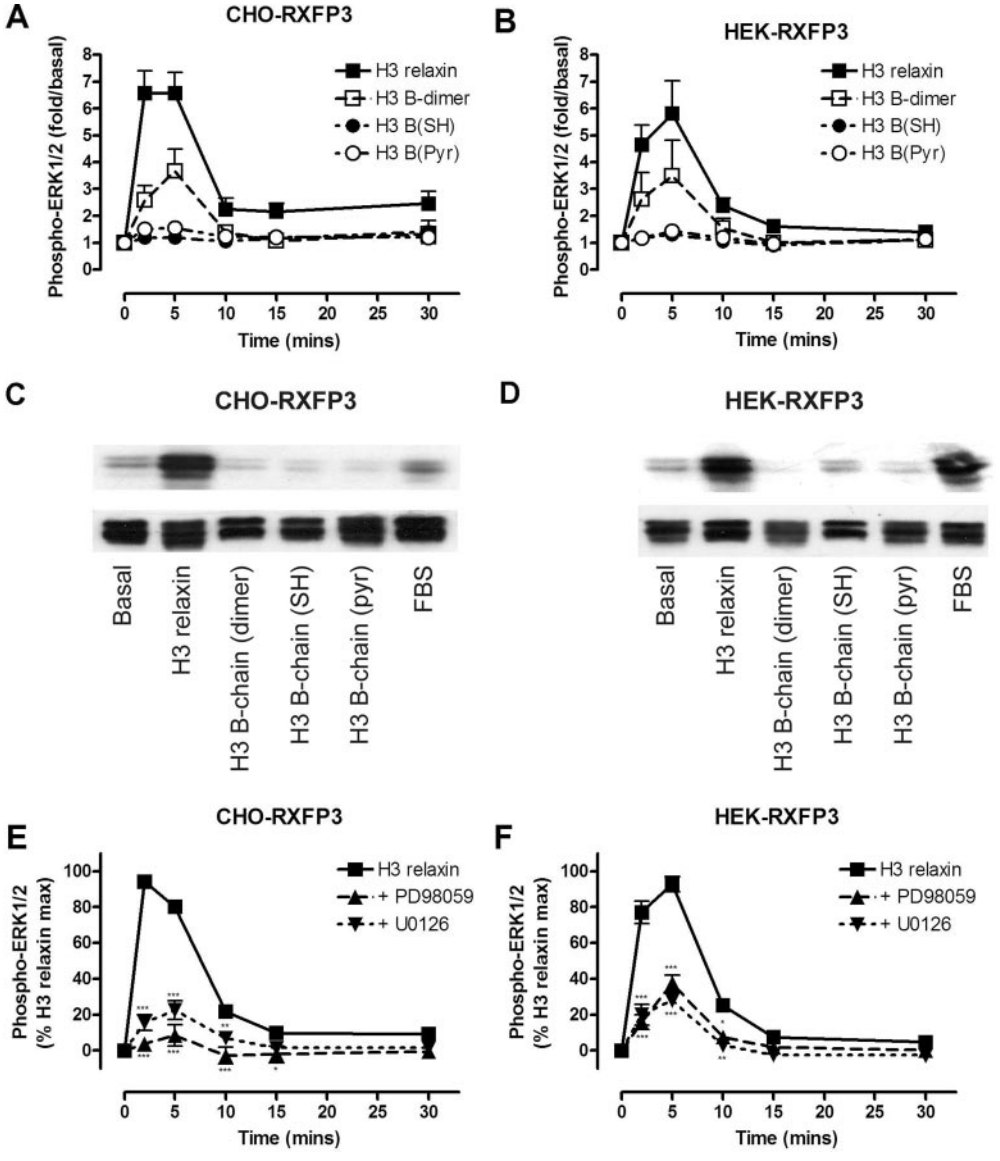

Fig. 3. Time course of ERK1/2 phosphorylation after activation of RXFP3 by relaxin family peptides. CHO-RXFP3 cells (A) and HEK-RXFP3 cells (B) were exposed to H3 relaxin peptides for periods of up to 30 min, and ERK activation was quantified using the phospho-ERK-specific Surefire AlphaScreen kit. Cells were treated with H3 relaxin, H3 relaxin B-chain peptides [single B-chain peptides with cysteine 10 protected by either an SH group H3 B(SH), or Pyr group H3 B(Pyr)] or H3 relaxin B-chain (dimer) (parallel dimer of two B-chains with one disulfide bond between position 10 cysteine and cysteine 22 attached to acetamidomethyl groups) Results are mean ± S.E.M. for six independent experiments performed in triplicate. ERK1/2 phosphorylation was also examined using Western blots of cell lysates from CHO-RXFP3 (C) and HEK-RXFP3 (D) cells after 5-min stimulation with relaxin family peptides (10 nM). Blots show phosphorylated ERK1/2 (top) (5-s exposure) and total ERK1/2 (bottom) (30-s exposure). Data are representative of three independent experiments. Pretreatment (30 min) of CHO-RXFP3 (E) and HEK-RXFP3 (F) with the MEK1/2 inhibitors PD98059 (20 μM) and U0126 (1 μM) blocked ERK1/2 phosphorylation. Data are mean ± S.E.M. for six independent experiments performed in triplicate. *, $p < 0.05$, **, $p < 0.01$, and ***, $p < 0.0001$, two-way ANOVA (E and F) with inhibitor treatment groups compared with H3 relaxin alone at each time point.

To confirm that ERK1/2 phosphorylation was dependent on activation of the upstream MEK1/2, we used two inhibitors of MEK1/2, U0126 (1 μM) and PD98059 (20 μM). In CHO-RXFP3 cells, ERK1/2 phosphorylation was inhibited by both compounds (PD98059, 88.0 ± 5.5%; U0126, 77.5 ± 5.3% of the peak response), indicating that ERK1/2 phosphorylation is dependent on MEK1/2 activation in these cells (Fig. 3E). Likewise, ERK1/2 phosphorylation was inhibited in HEK-RXFP3 cells (PD98059, 68.5 ± 7.1%; U0126, 72 ± 3.9%) (Fig. 3F). These concentrations of PD98059 and U0126 produced a similar inhibition of ERK1/2 phosphorylation after 30-min exposure to FBS [10% (v/v)]; $n = 4$; data not shown). Responses to FBS could be abolished by very high concentrations of MEK inhibitors (300 μM PD98059 and 100 μM U0126); however, at these concentrations, there is also likely to be an increase in nonspecific effects of the inhibitors.

RXFP3 Activates ERK1/2 via a Pathway Involving $G_{i/o}$ Proteins. We embarked on an inhibitor study to delineate the pathway to ERK activation. These inhibitors were tested for toxicity on the cells under the same conditions used in the ERK1/2 phosphorylation assays, by trypan blue exclusion; none of the inhibitors were toxic to CHO-RXFP3 or HEK-RXFP3 cells ($n = 4$; data not shown). Given our findings that RXFP3 inhibits forskolin-stimulated cAMP accumulation (Fig. 2A), presumably via interaction with $G_{i/o}$ proteins, we sought to examine whether RXFP3 activation of ERK1/2 is also dependent on $G_{i/o}$ proteins. CHO-RXFP3 and HEK-RXFP3 cells were treated with PTX (100 ng/ml, 18 h) and stimulated with H3 relaxin (10 nM). In the PTX-pretreated cells, there was >90% inhibition of ERK1/2 phosphorylation compared with H3 relaxin alone (Fig. 4, A and B), demonstrating a $G_{i/o}$-dependent mechanism of ERK1/2 activation.

Involvement of Epidermal Growth Factor Receptor Transactivation in RXFP3 Activation of ERK1/2. Epidermal growth factor (EGF) receptors are expressed endog-

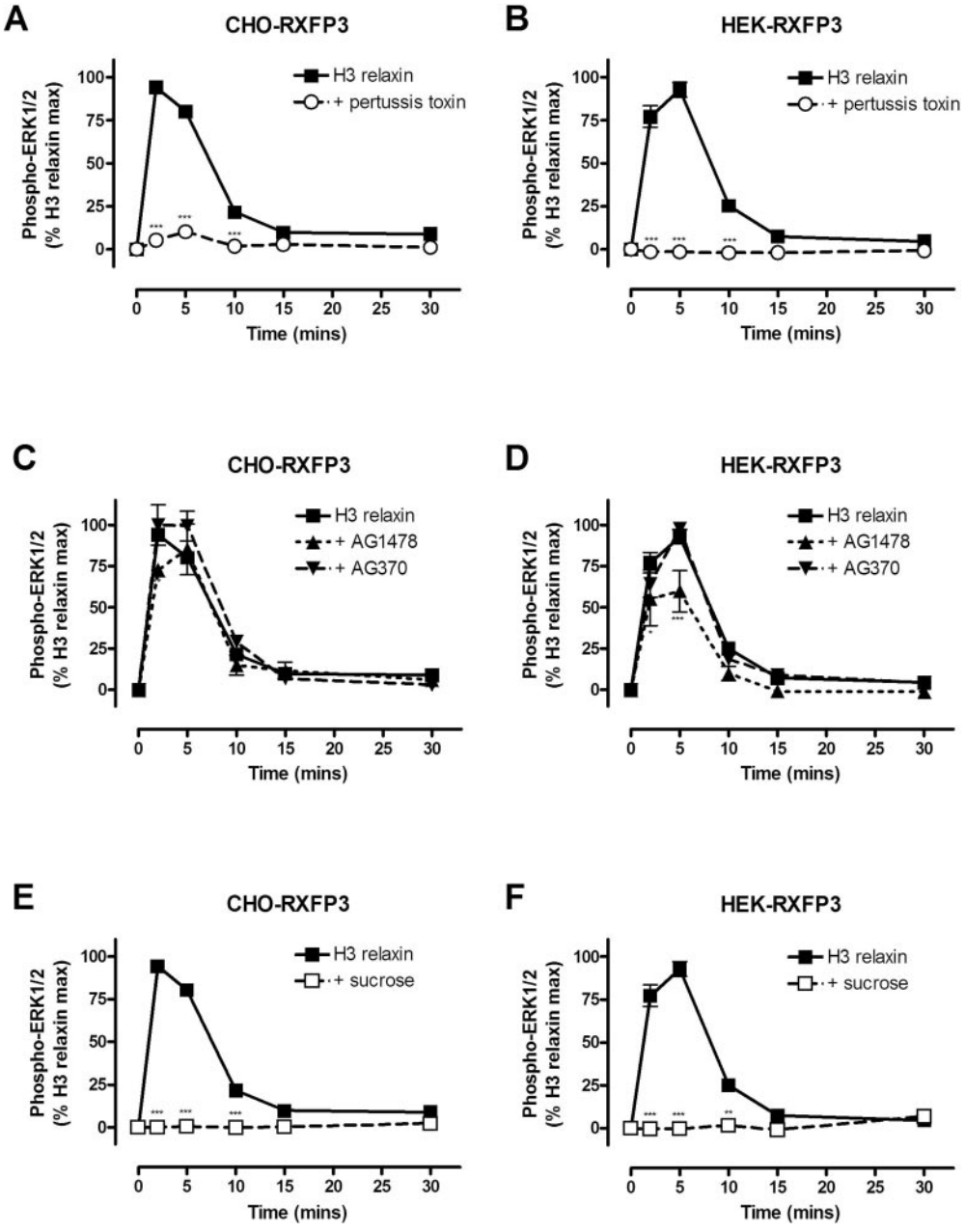

Fig. 4. ERK1/2 activation by H3 relaxin acting on RXFP3 receptors involves interaction with $G_{i/o}$ and receptor internalization with partial EGF receptor transactivation. ERK1/2 phosphorylation was measured after pretreatment with the $G_{i/o}$ protein inhibitor pertussis toxin (100 ng/ml, 18 h) in CHO-RXFP3 (A) and HEK-RXFP3 cells (B). To examine whether the ERK1/2 response involved tyrosine kinase receptor transactivation, CHO-RXFP3 (C) and HEK-RXFP3 (D) cells were treated with inhibitors of the EGF receptor (AG1478; 100 nM) and PDGF receptor (AG370; 50 μM) for 30 min before and during the assay. To study the role of receptor internalization, CHO-RXFP3 (E) and HEK-RXFP3 (F) cells were treated with the internalization inhibitor sucrose (450 mM) for 30 min before and during the assay. Data are mean ± S.E.M. for 5 to 15 independent experiments performed in triplicate. *, $p < 0.05$, **, $p < 0.01$, and ***, $p < 0.0001$, two-way ANOVA with inhibitor treatment groups compared with H3 relaxin alone at each time point.

enously in HEK293 cells but are absent from CHO-K1 cells (Shi et al., 2000). An EGF receptor inhibitor, tyrphostin AG1478 (100 nM) was used to investigate whether EGF receptor transactivation contributed to ERK1/2 activation downstream of H3 relaxin-stimulated RXFP3. Furthermore, an inhibitor of PDGF receptor autophosphorylation, tyrphostin AG370 (50 μM) was also used to examine possible transactivation of the PDGF receptor because ERK1/2 phosphorylation has been reported to occur in CHO-K1 cells by this mechanism (Oak et al., 2001). In HEK-RXFP3 cells, AG1478 inhibited H3 relaxin-stimulated (5 min) ERK1/2 phosphorylation by 40.1 ± 12.7% (Fig. 4D) but had no significant effect on the 5-min response in CHO-RXFP3 cells (Fig. 4C). AG370 did not inhibit ERK1/2 phosphorylation in either cell line (Fig. 4, E and F).

RXFP3 Activation of ERK Depends on Internalization Processes. The activation of ERK1/2 by GPCRs sometimes requires the internalization of membrane-bound proteins (Werry et al., 2005b). To investigate whether RXFP3-mediated ERK1/2 activation is dependent on either endocytosis or lipid rafts, we used hypertonic sucrose, an inhibitor of clathrin-coated pit-mediated endocytosis to disrupt the internalization machinery (Hansen et al., 1993; Audet et al., 2005). Sucrose removes clathrin-coated pits from the plasma membrane and transforms them into clathrin cages that exist in the cytosol (Hansen et al., 1993). Treatment of cells with sucrose (450 mM, 30 min) (Hansen et al., 1993) abolished ERK phosphorylation in both cell lines, suggesting that internalization and/or localization into lipid rafts or signaling platforms was required for ERK1/2 signaling to occur (Fig. 4, E and F).

RXFP3 Activation of ERK1/2 Requires PKC. The pertussis toxin-sensitive Gα_o subunit can directly activate PKC, resulting in ERK1/2 phosphorylation in CHO and COS cells (van Biesen et al., 1996). Therefore, two nonselective inhibitors of PKC were used to determine whether RXFP3 activation of an isoenzyme of PKC was involved in the pathway leading to ERK1/2 phosphorylation. Ro 31-8220 (bisindolylmaleimide derivative of staurosporine) and chelerythrine chloride are nonselective PKC inhibitors that inhibit conventional (α, βI, βII, γ), novel (δ, ε, η, θ), and atypical (ι/λ, μ, ζ) isoenzymes of PKC by competing for the ATP binding site. Both inhibitors caused ~80% inhibition of the H3 relaxin-mediated ERK1/2 phosphorylation in CHO-RXFP3 cells, whereas Ro 31-8220 inhibited 98.2 ± 5.2% of the response and chelerythrine inhibited 65.0 ± 7.8% of the response in HEK-RXFP3 cells (Fig. 5, A and B).

To determine the isoenzymes of PKC involved in RXFP3 activation of ERK1/2, a range of PKC inhibitors with greater selectivity between isoenzymes were used. Bisindolylmaleimide I and prolonged PMA exposure inhibit both conventional and novel PKC, Gö6976 inhibits conventional PKC isoenzymes, and PKCζ pseudosubstrate inhibits atypical PKC. Treatment of CHO-RXFP3 cells with PMA (200 nM, 24 h), bisindolylmaleimide I (1 μM, 30 min), or chelerythrine chloride (10 μM, 30 min) significantly inhibited RXFP3-mediated ERK1/2 phosphorylation, as did the presence of PKCζ pseudosubstrate (10 μM, 30 min) (Fig. 5C). This pattern of inhibition was also seen in HEK-RXFP3 cells, although only chelerythrine blocked the response to a statistically significant degree (Fig. 5D). However, the trends observed in both cell lines suggest that both novel and atypical isoforms of PKC are involved in RXFP3-mediated ERK1/2 activation in these cells. Gö6976 had minimal effect on ERK1/2 activation, indicating that conventional PKC isoforms play little role in the response.

RXFP3 Activation of ERK1/2 Involves Phospholipase C β and Raf Activation. Conventional and novel PKC isoforms are activated when phospholipase C β (PLCβ) converts membrane phospholipids into diacylglycerol. The PLCβ inhibitor U73122 (10 μM) and the PLCβ/Raf inhibitor ET-18-OCH_3 (100 μM) were used to investigate whether PLCβ and Raf activation were involved in RXFP3 activation of ERK1/2. ET-18-OCH_3 completely blocked H3 relaxin-mediated ERK1/2 phosphorylation in both cell lines. It also completely blocked serum-mediated ERK1/2 activation in our cell lines (n = 4; data not shown), suggesting that ET-18-OCH_3 blocked ERK1/2 phosphorylation by directly inhibiting Raf (Sammader et al., 2003) rather than by inhibiting PLCβ. U73122 inhibited ERK1/2 phosphorylation by 35.6 ± 8.1% in CHO-RXFP3 cells at 2 min and by 45.0 ± 4.9% in HEK-RXFP3 cells at 5 min (Fig. 5, E and F), suggesting that a small component of ERK1/2 activation occurs via a PLCβ-dependent pathway in both cell lines.

RXFP3 Activation of ERK Involves Phosphatidylinositol 3-Kinase and Src. GPCRs that are coupled to PTX-sensitive G proteins often activate ERK1/2 via $G_{\beta\gamma}$ subunits, which directly activate phosphatidylinositol 3-kinase (PI3K), Src family tyrosine kinases and many of the intermediates shown to be important in receptor tyrosine kinase-mediated ERK1/2 phosphorylation (Werry et al., 2005b). Wortmannin and LY294002 are commonly used inhibitors of PI3K that completely inhibit PI3K activation at the concentrations used in this study (Davies et al., 2000). Wortmannin (100 nM) and LY294002 (50 μM) caused significant reductions in H3 relaxin-stimulated ERK1/2 phosphorylation in both the CHO-RXFP3 and HEK-RXFP3 cells (Fig. 6, A and B). In addition, the Src family tyrosine kinase inhibitors, PP1 (1 μM) and PP2 (10 μM) caused a comparable inhibition of H3 relaxin-stimulated ERK response (Fig. 6, C and D), suggesting that both PI3K and Src tyrosine kinases play a role in RXFP3-mediated ERK activation.

H3 Relaxin Activates ERK1/2 in SN56 Cells. The murine septal neuron cell line SN56 endogenously expresses RXFP3 receptors, as detected by reverse transcription-polymerase chain reaction (n = 2; data not shown). Reverse transcription-polymerase chain reaction also indicated that these cells do not express RXFP1, RXFP2, or RXFP4 receptors (n = 2; data not shown). Saturation binding studies revealed a single population of sites with a density of 43.33 ± 6.01 fmol/mg protein with pK_d = 9.72 ± 0.14 (apparent K_d = 0.19 nM) (n = 4). When stimulated with H3 relaxin there was a rapid and transient ERK1/2 activation with peak activation occurring between 2 to 5 min in these cells (maximum response, 13.13 ± 4.31-fold/basal; n = 4). ERK1/2 phosphorylation after 5-min stimulation with H3 relaxin was blocked by 95.2 ± 1.1% in the presence of PTX (100 ng/ml, 18 h); by 95.4 ± 0.6% in the presence of sucrose (450 mM, 30 min); by 87.7 ± 0.6% in the presence of Ro 31-8220 (10 μM, 30 min); and by 82.2 ± 0.6% with PD98059 (20 μM, 30 min) (Fig. 7). The EGF receptor inhibitor AG1478 blocked ERK1/2 phosphorylation by 62.0 ± 10.8% after 5-min stimulation with H3

relaxin (Fig. 7). These results are consistent with the results obtained in the CHO-RXFP3 and HEK-RXFP3 cell lines recombinantly expressing human RXFP3 receptors.

Discussion

The H3 relaxin/RXFP3 receptor system is selectively enriched in the central nervous system and plays a significant role in the stress response and the control of food intake (McGowan et al., 2005, 2006; Tanaka et al., 2005). Recent data suggest that activation of ERK1/2 may be important in central responses to both stress and feeding (Morikawa et al., 2004; Shen et al., 2004; Sutton et al., 2004a; Sasaguri et al., 2005). In this study, we provide, for the first time, direct evidence for robust activation of ERK1/2 after stimulation of the RXFP3 receptor by H3-relaxin in CHO-RXFP3, HEK-RXFP3, and SN56 cell lines, providing a potential mechanistic link between RXFP3 activation and its central effects.

We have compared three cellular backgrounds, CHO-K1 and HEK293, that recombinantly express human RXFP3 receptors and SN56 cells, a neuronally derived cell line, which endogenously express murine RXFP3 receptors. The receptor expression levels in the recombinant cell lines are similar to the level of receptor expressed in SN56 cells and are probably similar to levels found in the brain. The CHO-K1 and HEK293 cell lines were selected as models for assessing cell signaling pathways because they are commonly used cell lines in characterizing GPCR coupling to various signaling pathways and have been principally used for the evaluation of human RXFP3 receptors (Liu et al., 2003). The CHO and HEK cell lines also differ in expression of the EGFR, which is commonly recruited by GPCRs in signaling to ERK1/2; therefore, we could determine the potential effect of EGFR transactivation on ERK1/2 signaling by RXFP3 (Shi et al., 2000; Pierce et al., 2001; Werry et al.,

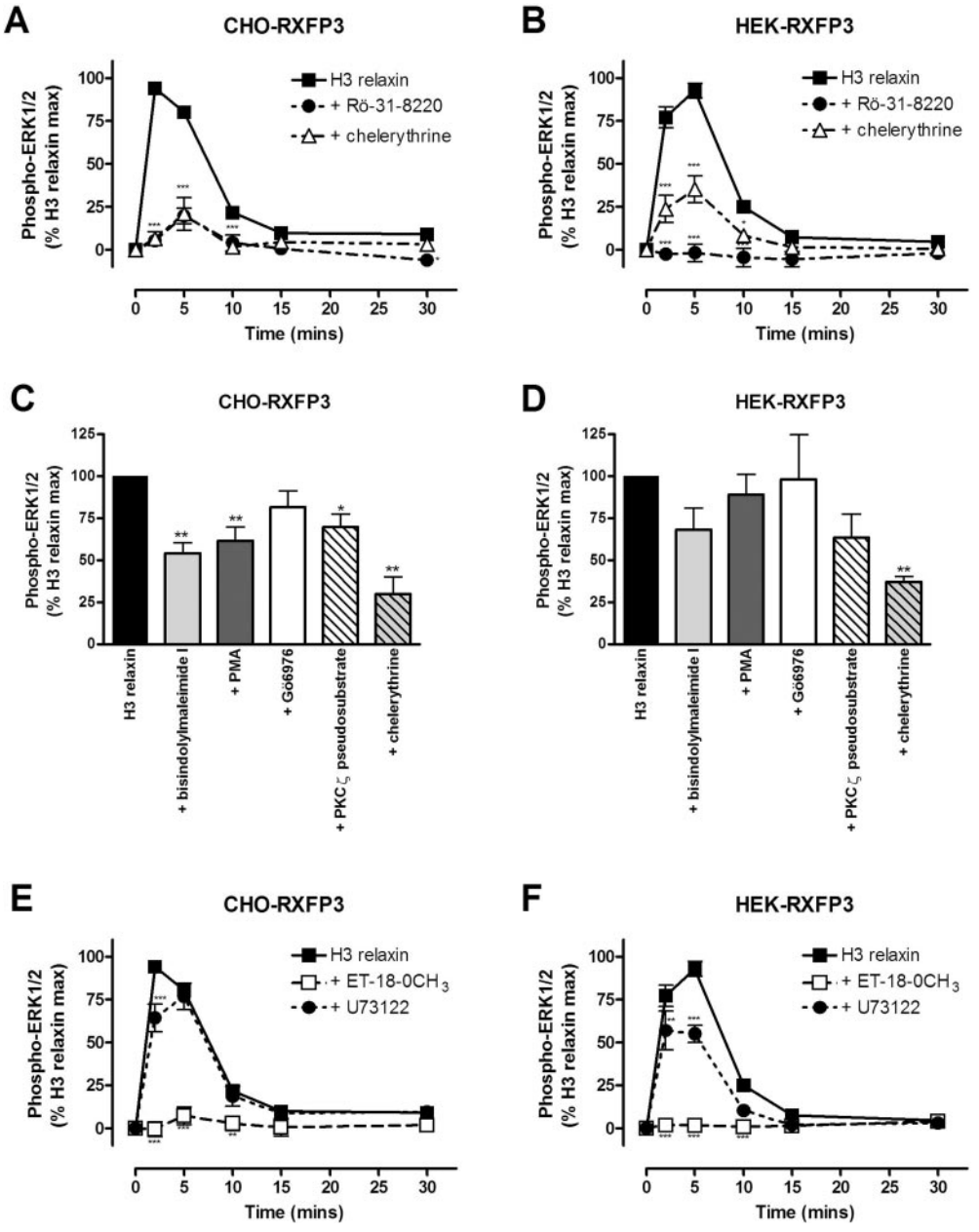

Fig. 5. Effect of PKC and PLC inhibitors on H3 relaxin-stimulated ERK1/2 phosphorylation. The time course of ERK1/2 phosphorylation in CHO-RXFP3 (A) and HEK-RXFP3 cells (B) is shown in the presence and absence of non-isoenzyme-specific PKC inhibitors Ro 31-8220 (10 μM) and chelerythrine (10 μM). Cells were treated with PKC inhibitors for 30 min before and during H3 relaxin stimulation. ERK1/2 phosphorylation in CHO-RXFP3 (C) and HEK-RXFP3 (D) cells in response to H3 relaxin (10 nM; 5 min) is also shown in the presence of PKC isoenzyme-specific inhibitors bisindolymaleimide I (conventional and novel PKC) (1 μM, 30 min), Gö6976 (conventional PKC) (10 μM, 30 min), PMA (conventional and novel PKC) (200 nM, 24 h), and PKCζ pseudosubstrate (atypical PKC) (10 μM, 30 min). ERK1/2 phosphorylation in CHO-RXFP3 cells (E) and HEK-RXFP3 cells (F) in the presence of PLCβ inhibitors U73122 (10 μM) and ET-18-OCH_3 (100 μM). Cells were treated with PLC inhibitors for 30 min before and during H3 relaxin stimulation. Data are mean ± S.E.M. of 6 to 15 independent experiments performed in triplicate. *, $p < 0.05$, **, $p < 0.01$, and ***, $p < 0.0001$, two-way ANOVA (A, B, E, and F) or one-way ANOVA (C and D) with inhibitor treatment groups compared with H3 relaxin alone.

2005b). In the absence of a human neuronal cell line that endogenously expresses human RXFP3 receptors, murine SN56 cells endogenously expressing murine RXFP3 receptors were also examined to determine whether the pathways elucidated in recombinant expression systems remained relevant in the context of a neuronal phenotype.

The pharmacological profile of the CHO-RXFP3 and HEK-RXFP3 cell lines for competition of [^{125}I]INSL5/H3 relaxin and for inhibition of forskolin-stimulated cAMP was similar to that characterized previously in transfected cell lines and in brain tissue (Liu et al., 2003; Sutton et al., 2004b), with responses observed with H3-relaxin and a dimer of the B-chain of H3-relaxin but not other relaxin peptides. It is interesting that synthetic monomeric H3-relaxin B-chains (with the 10th cysteine residue protected by either an SH group or a Pyr group) were ineffective, whereas recombinantly expressed B-chain monomers retain activity (Liu et al., 2003). This difference could relate to the structure of the synthetic peptides in solution compared with the H3 relaxin B-chain folded in vivo; however, the structures of the synthetic H3 relaxin B-chains have not been determined. It is interesting that the lack of activity by the monomeric B-chain peptides at RXFP3 was overcome upon formation of the dimer.

Previous work from our laboratory demonstrated that the MEK inhibitor PD98059 could modify the acidification response of CHO-RXFP3 cells, implying that RXFP3 receptors could signal to the ERK1/2 pathway (van der Westhuizen et al., 2005). This was confirmed in the current study by direct measurement of ERK1/2 phosphorylation in CHO-RXFP3, HEK-RXFP3, and SN56 cells. The primary mechanism of activation was conserved across the cellular backgrounds with responses abolished by PTX, illustrating that the effect was downstream of activation of the $G_{i/o}$ family of proteins and by direct inhibition of MEK, the principal kinase responsible for ERK1/2 phosphorylation (Fig. 8). It is interesting that activation of ERK1/2 was also completely abolished in all three cell lines by hypertonic sucrose, suggesting that clustering of the receptors into clathrin-coated pits and/or internalization of RXFP3 was prerequisite for recruitment of signaling proteins, although further studies are required to examine the mechanism of RXFP3 receptor internalization. The phosphorylation of ERK1/2 was also dependent on activation of PKC, with nonspecific inhibition of PKC isoenzymes completely blocking the ERK response in the three cell lines. Examination of the contribution of different classes of PKC (conventional, novel, and atypical) through the use of more specific inhibitors revealed both novel and atypical PKC isoenzymes in the observed ERK response, with little contribution from conventional PKC isoenzymes. This latter finding is not surprising, because activation of conventional PKC isoenzymes requires Ca^{2+}, whereas activation of RXFP3 re-

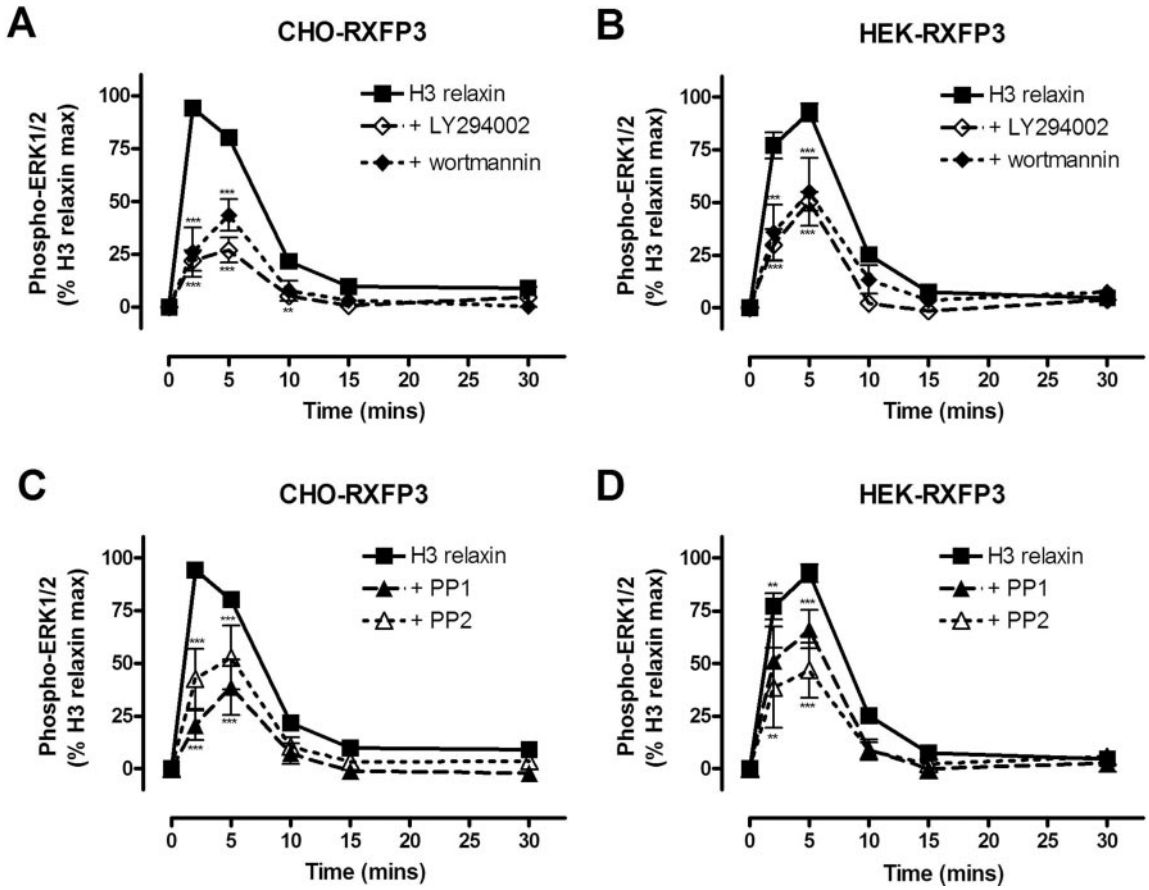

Fig. 6. Effect of PI3K and Src family tyrosine kinase inhibitors on H3 relaxin-stimulated ERK1/2 phosphorylation. ERK1/2 phosphorylation in CHO-RXFP3 (A) and HEK-RXFP3 cells (B) was examined in the presence and absence of the PI3K inhibitors wortmannin (100 nM) and LY294002 (50 μM). ERK1/2 phosphorylation was also studied in CHO-RXFP3 (C) and HEK-RXFP3 cells (D) in the presence and absence of the Src inhibitors PP1 (1 μM) and PP2 (10 μM). Cells were treated with PI3K or Src inhibitors for 30 min before and during H3 relaxin stimulation. Data are mean ± S.E.M. of 6 to 15 independent experiments performed in triplicate. *, $p < 0.05$, **, $p < 0.01$, and ***, $p < 0.0001$ two-way ANOVA with inhibitor treatment groups compared with H3 relaxin alone at each time point.

ceptors does not lead to coupling to $G_{q/11}$ proteins and mobilization of intracellular Ca^{2+} (Liu et al., 2003). The differences in PKC isoenzyme-dependence between the two recombinant cell types is probably due to variation in endogenous levels of novel and atypical PKCs and/or the types of $G_{i/o}$ proteins naturally expressed within the cells. $G_{\alpha o}$ can directly activate PKC (van Biesen et al., 1996), whereas $G\beta\gamma$ subunits of $G_{i/o}$ proteins are known to activate PLCβ (Dickenson and Hill, 1998), leading to the formation of IP_3 and diacylglycerol, the latter being required for direct activation of novel PKC isoenzymes. Assessment of the contribution of PLCβ to the response supported different contributions of this enzyme to responses for the CHO and HEK cell lines. Inhibition of PLCβ with U73122 caused ~45% reduction in peak ERK activation in HEK-RXFP3 cells but had only a very weak effect at the 2-min time point in CHO-RXFP3 cells, again suggesting that there are differences in the PKC isoenzymes endogenously expressed and activated in the two recombinant cell lines.

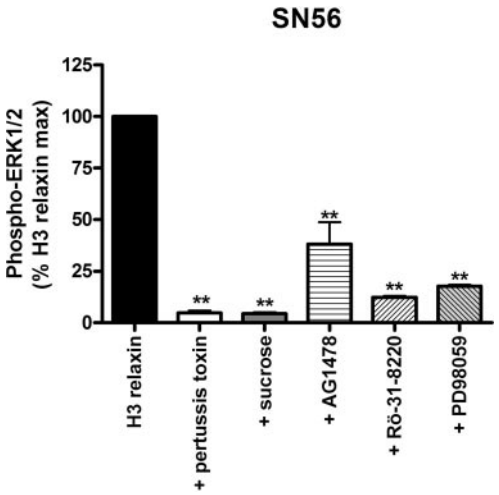

Fig. 7. Effect of pertussis toxin, sucrose, AG1478, Ro 31-8220, and PD98059 on H3 relaxin-stimulated ERK1/2 phosphorylation in SN56 cells. ERK1/2 phosphorylation was measured in neuronally derived SN56 cells after pretreatment with the $G_{i/o}$ protein inhibitor pertussis toxin (100 ng/ml, 18 h), the EGFR inhibitor tyrphostin AG1478 (100 nM, 30 min), the internalization inhibitor sucrose (450 mM, 30 min), the PKC inhibitor Ro 31-8220 (10 μM, 30 min), and the MEK1/2 inhibitor PD98059 (20 μM, 30 min). Cells were treated with inhibitors for the indicated times before stimulation with H3 relaxin and were present for the duration of the time course. Data are mean ± S.E.M. of four independent experiments performed in triplicate. **, $p < 0.01$ (one-way ANOVA with inhibitor treatment groups compared with H3 relaxin alone by Dunnett's multiple comparison post hoc test).

Inhibition of either PI3K or Src kinase produced a ~50% depression in phosphorylation of ERK1/2 in both CHO and HEK cell lines, implicating activation of PI3K and downstream signaling effectors in the RXFP3-mediated ERK activation pathway. Recruitment of this pathway is commonly seen for $G_{i/o}$-coupled GPCRs, in which activation may occur through a $G\beta\gamma$ subunit-dependent mechanism or indirectly via PKC (Werry et al., 2005b) (Fig. 8). Partial inhibition of ERK activation by PI3K inhibitors is also seen for endothelin-1 ET_A and thromboxane prostanoid receptors; however, these receptors can promiscuously couple to both $G_{i/o}$ and $G_{q/11}$ proteins to activate multiple signaling pathways (Robin et al., 2002; Citro et al., 2005). In the case of the RXFP3 receptor, the ERK response is entirely downstream of $G_{i/o}$ proteins, with the divergence in activation pathways probably caused by the direct activation of Ras/Raf by PKC in addition to the role of PI3K (van Biesen et al., 1996; Mannon and Raymond, 1998; Audet et al., 2005) (Fig. 8). In the HEK293 cells that express an endogenous EGF receptor, there was a significant component of the response that arose via transactivation of the EGF receptor with an ~40% inhibition observed with tyrphostin AG1478, suggesting that in cells which express both RXFP3 and EGF receptors, ERK1/2

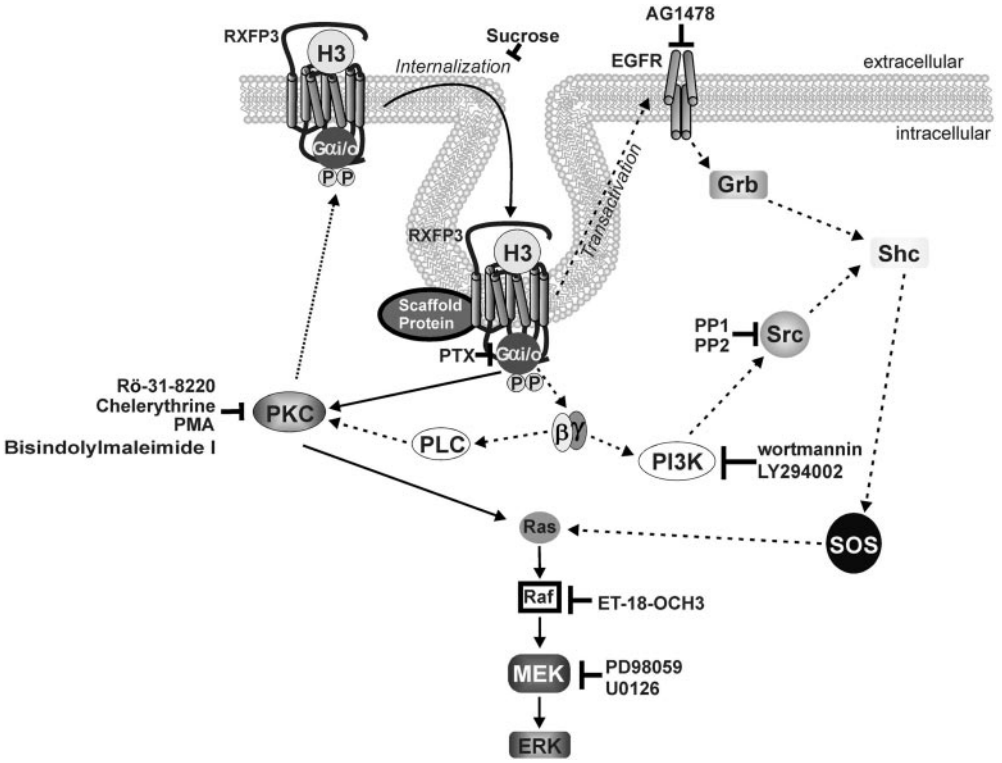

Fig. 8. Proposed pathways involved in ERK1/2 phosphorylation after stimulation of RXFP3 receptors with H3 relaxin. ERK1/2 is activated in CHO-RXFP3 and HEK-RXFP3 cells by H3 relaxin via a $G\alpha_{i/o}$ protein. The RXFP3 receptor is then internalized or localized to a lipid-rich signaling platform. The $G\beta\gamma$-subunits of $G\alpha_{i/o}$ may act directly on PI3K, resulting in partial activation of ERK1/2. The $G\beta\gamma$-subunits weakly activate PLCβ in CHO-RXFP3 cells; however, this activation is much stronger in HEK-RXFP3 cells. In CHO-RXFP3 cells, it is more likely that $G\alpha_o$ directly activates PKC. Novel and/or atypical PKC isoenzymes seem to be activated to different extents in the two cell lines. Once activated, PKC could directly activate the Ras, Raf, MEK1/2, and ERK1/2 pathway and may also activate the PI3K pathway. In HEK-RXFP3 cells EGFR transactivation is required for part of the total ERK1/2 response. Pathways that must be activated for ERK1/2 phosphorylation to occur are shown with solid lines. Partially activated pathways are shown with broken lines, and speculative pathways are indicated with dotted lines.

phosphorylation will occur, in part, via this pathway. When SN56 cells were treated with this inhibitor, there was ~62% inhibition of the H3 relaxin-mediated ERK1/2 response, confirming that in cells which endogenously express both RXFP3 and EGFR, part of the ERK1/2 activation is likely to arise via EGFR transactivation. However, there is still a component of the response that is independent of this transactivation event.

As described above, nonspecific inhibition of PKC isoenzymes or clathrin-mediated clustering/internalization led to the abolition of the ERK1/2 response. The mechanistic link between these two events is not clear; however, it is possible that the recruitment of scaffolding proteins (such as arrestin or dynamin) (Tohgo et al., 2003) into clathrin-coated pits is required to trigger the signaling cascades observed in the current study. An alternate hypothesis is that PKC-dependent phosphorylation of the receptor is required to trigger accessory protein recruitment and internalization that lead to the activation of downstream signaling pathways. Bioinformatic analysis of the RXFP3 sequence (Minimotif Miner) (Balla et al., 2006) indicates the presence of potential PKC phosphorylation sites in the carboxy-terminal tail and intracellular loops 2 and 3 of the receptor, although no direct evidence is currently available on whether these sites are phosphorylated or what are the consequences of PKC phosphorylation of the receptor.

In conclusion, we have demonstrated coupling of the H3-relaxin-stimulated RXFP3 receptor to ERK phosphorylation and that the signaling pathways leading to ERK activation are well-conserved between CHO-RXFP3 and HEK-RXFP3 cells. ERK activation occurs via PKC or PI3K and also involves transactivation of the EGF receptor, in which this receptor is coexpressed. Because the results with PTX, sucrose, AG1478, Ro 31-8220, and PD98059 were also reproducible in neuronally derived SN56 cells; it is likely that the ERK1/2 pathways described here are important in the brain. G_i and G_o proteins are both expressed in neurons, with G_o proteins widely expressed throughout the brain (McIntire et al., 1999) and G_i proteins most strongly expressed in the frontal cortex (Young et al., 1993). This work provides a potential mechanistic link between RXFP3 activation and its effects on stress responses and food intake that can be evaluated in neuronal systems. The comparative data from CHO versus HEK cells indicate that differential expression of G_i versus G_o proteins is likely to alter the mechanism and strength of ERK1/2 activation.

Acknowledgments

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Address correspondence to: Dr. Roger J. Summers, Department of Pharmacology, PO Box 13E, Monash University, Clayton, VIC 3800, Australia. E-mail: roger.summers@med.monash.edu.au