Arrestin-mediated ERK Activation by Gonadotropin-releasing Hormone Receptors

**RECEPTOR-SPECIFIC ACTIVATION MECHANISMS AND COMPARTMENTALIZATION**

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Activation of seven-transmembrane region receptors typically causes their phosphorylation with consequent arrestin binding and desensitization. Arrestins also act as scaffolds, mediating signaling to Raf and ERK and, for some receptors, inhibiting nuclear translocation of ERK. GnRHR receptors (GnRHRs) act via Gq/11 to stimulate desensitization. Arrestins also act as scaffolds, mediating signaling.

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The abbreviations used are: GnRH I, mammalian gonadotropin-releasing hormone; GnRHR, GnRH receptor; [His5,Trp7,Tyr8]GnRH, the best characterized GnRHR (GnRHR). The best characterized GnRHRs (type I mammalian GnRHRs) are selective for GnRH I and, unlike all other known 7TM receptors, lack C-terminal cytoplasmic domains (2, 3). All other GnRHRs (type II, type III, and nonmammalian type I GnRHRs) have higher affinity for GnRH II than for GnRH I and possess intracellular C-tails of varying length (3, 4), raising the question of the functional relevance of these structures in GnRHRs. Sustained activation of 7TM receptors typically causes their rapid homologous desensitization, a process involving their phosphorylation within the receptor’s C-tail and/or third intracellular loop by G-protein-coupled receptor kinases. This facilitates arrestin binding, which reduces G-protein activation and targets the desensitized receptor for internalization. 7TM receptor internalization is typically via clathrin-coated vesicles that are pinched off from the plasma membrane by a dynamin collar (5, 6). This general model appears applicable to nonmammalian (C-tailed) GnRHRs that (where studied) undergo rapid and arrestin-dependent homologous desensitization and internalization, whereas type I mammalian GnRHRs do not. Work with truncated, chimeric, and mutated receptors supports the idea that the unique absence of C-tails from these receptors explains their resistance to desensitization and slow rate of internalization (4, 7–13). We have recently shown that internalization of the human GnRHR (hGnRHR) is insensitive to a dominant negative K44A dynamin mutant, whereas that of the C-tailed Xenopus GnRHR (XGnRHR) is dynamin-dependent (10), apparently because arrestin binding to the C-tail targets the XGnRHR for dynamin-dependent internalization (14, 15).

Many 7TM receptors activate the three-tiered Raf/MEK/ERK MAPK pathway. In resting cells, MEK and ERK exist as a complex in the cytoplasm, but, upon stimulation, MEK phosphorylates ERK on Thr202 and Tyr204, causing concomitant dissociation from MEK and translocation to the nucleus (14, 15). Although ERK has no nuclear export sequence, activated protein kinase; ERK, extracellular signal-regulated kinase; pp-ERK1/2, Thr202/Tyr204 dual phosphorylated ERK1 and/or ERK2; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase; DMEM, Dulbecco’s modified Eagle’s medium; Ad, adenovirus; 3F, triple FLAG; HA, hemagglutinin; PBS, phosphate-buffered saline; EGF, epidermal growth factor; PDBo, phorbol 12,13-dibutyrate; IP3, inositol phosphates; p puff, plaque-forming units.
GnRH Receptor Signaling to ERK

its dephosphorylation within the nucleus enables reassociation with MEK (which does have a nuclear export sequence) and consequent trafficking to the cytoplasm (18, 19). Several scaffold proteins are known to influence the cellular distribution of activated ERK so that distinct stimuli causing comparable increases in whole cell ERK activation can have different effects on ERK1/2 translocation to the nucleus and gene expression (20, 21). In this regard, it has been found that arrestins can scaffold several MAPK proteins (including ERK) and attention has focused on the possible relevance of such scaffolding for 7TM receptor function (22, 23). For example, activation of the AT1a angiotensin, V2 vasopressin, or PAR2 thrombin receptors causes arrestin-mediated desensitization and receptor internalization but also causes formation of a complex comprising receptors, arrestin, and ERK. Such complexes are thought to inhibit nuclear translocation of ERK, thereby preventing trafficking to nuclear targets and favoring cytoplasmic signaling (24–27). However, this does not mean that arrestin-mediated sequestration of ERK necessarily results in cytoplasmic scaffolding. 7TM receptors have an additional plethora of signaling routes to ERK independent of arrestin and Gα subunits, including cross-talk between other 7TM receptors and receptor or nonreceptor tyrosine kinases, which can vary considerably according to cell type and biological context (28). The hGnRHR, for example, causes transient EGF receptor-mediated ERK1/2 activation in GT1-7 neurons, whereas in HEK293 and LBT2 pituitary cells, activation is largely PKC-mediated (29, 30).

The data outlined above demonstrate that the molecular evolution of GnRHRs (the advent of tailless receptors with mammals) is functionally relevant in terms of termination of signaling (desensitization and internalization). Here we address the possibility that it also influences the direction of signaling. Specifically, we hypothesized that binding of arrestins to the C-terminal tails of Xenopus (but not human) GnRHRs would influence the mechanisms, kinetics, and compartmentalization of ERK1/2 activation. We show that arrestin(s) can, indeed, mediate signaling of XGnRHRs to ERK, an effect revealed by inhibition of PKC-mediated ERK activation and not seen with hGnRHRs. Surprisingly, we find that the hGnRHR is less efficient than the XGnRHR at causing nuclear translocation, implying that scaffolds other than arrestins play a major role in hGnRHR-mediated ERK compartmentalization.

EXPERIMENTAL PROCEDURES

Materials—GnRH and chicken GnRH II (GnRH II) were purchased from Sigma. Buserelin and \[^{125I}\]buserelin ([T-BuSer\(^6\),Pro\(^9\)-NH-ethylamide] GnRH; 2000 Ci/mmol) were provided by Prof. J. Sandow (Aventis Pharma GmbH, Frankfurt, Germany). \[^{125I}\]GnRH II (~3400 Ci/mmol as determined by self-displacement) was prepared as described (14). Culture media, sera, and plasticware were from Invitrogen (Crawley, UK). cDNAs encoding wild-type GnRHRs (human and Xenopus) were kindly provided by Prof. R. Millar (Medical Research Council Human Reproductive Sciences Unit, Edinburgh, UK), arrestin-GFP and \(\Delta\)arrestin-(319–418) cDNAs were a gift from Prof. J. L. Benovic (Thomas Jefferson University, Philadelphia, PA), and the pEXV3MEK1 construct was a gift from Prof. C. Marshall (Cancer Research UK, London, UK).

Engineering of Plasmids and Viruses—XGnRHR and hGnRHR cDNAs were commonly used within the pCR3.1 expression vector (Invitrogen). The XGnRHR was tagged with the nonapeptide (Tyr(P)-Pro-Tyr-Asp-Val-Pro-Asp-Tyr-Ala-NH\(_2\)) hemagglutinin (HA) tag derived from the influenza virus. The first primer (5’-AAA GGA TCC CACC ATG TAC CCA TAC GAC GTC CCA GAC TAC GCT GCA GTA AAT CAA ACT CAG AGA T-3’) was designed against the 5’-end of the XGnRHR sequence. The recognition site for the restriction enzyme BamHI was placed first (underlined), prior to the Kozak sequence, the ATG initiation codon, and the HA tag sequence (italic type), as well as the first 20 bases of the XGnRHR 5’-end. The second primer (5’-CTG ATG CTG CAG AGA ACA GC-3’) was designed as the complementary sequence to a coding region of XGnRHR including PstI (underlined). The product of the PCR was then subcloned into a corresponding digest of XGnRHR in pCR3.1. 3X FLAG-tagged hGnRHR (3F hGnRHR) was generated by in-frame subcloning of an Nsil digest of hGnRHR in pCMVZE0 (Invitrogen) into a PstI digest of pX3FLAG-CMV7 (Sigma). The pERK2-GFP construct was prepared as described (24). Sequences encoding arrestin-2-GFP and arrestin-3-GFP, \(\Delta\)arrestin-(319–418), hGnRHR, and XGnRHR were subcloned into the adenovirus (Ad) transfer vectors pXCCCMV (XGnRHR) or pXCXCMV-WPRE (all others). The recombinant Ad were then generated by homologous recombination with the plM17 backbone vector (Microbix Systems Inc., Toronto, Canada) in HEK293 cells, grown to high titer, and then purified by CsCl density gradient centrifugation as described (9, 10).

Cell Culture and Transfection—HeLa cells were cultured in 10% FCS-supplemented Dulbecco’s modified Eagle’s medium (DMEM). For experiments, cells were harvested by trypsinization, plated in DMEM supplemented with 2% FCS, and incubated for 24–48 h in culture plates prior to the assay. In some experiments, cells were transfected by infection with recombinant Ad-expressing GnRHRs, as described (9). The Ad-containing medium was removed after ~4–6 h and replaced with fresh DMEM supplemented with 2% or 0.1% FCS (Figs. 1 and 2–10, respectively), as indicated in the figure legends. The cells were then maintained for 16–48 h in culture prior to assay. Alternatively, they were transiently transfected with pCR3.1 vectors encoding GnRHRs with or without arrestin-2-GFP, arrestin-3-GFP, or \(\Delta\)arrestin-(319–418) using Superfect and following the manufacturers’ instructions. These cells were also maintained in culture for a further 16–24 h prior to use in assays as indicated in the figure legends. In these cells, transfection efficiencies with recombinant Ad are typically >90% and greatly exceed transfection rates with conventional calcium phosphate- or lipid-based procedures (10). We therefore routinely use Ad for transfection when end points using the entire cell population are monitored (e.g. \[^{3}H\]IP\(_x\) assays and Western blotting) and conventional plasmid-based transfection for experiments in which individual cell responses are measured (e.g. immunohistochemistry and ERK-GFP translocation) as indicated below. Where investigated, we have found no effect of transfection method (Ad versus plasmid) on functional characteristics of transfected receptors or effectors (15) (data herein).

Radioligand Binding and \[^{3}H\]IP\(_x\) Assays—Radioligand binding to cell surface GnRHRs was quantified using whole cell binding assays with cells grown in 24-well plates and incubated at 21 ℃ with cognate ligand ([\(^{125I}\)]buserelin for hGnRHRs and [\(^{125I}\)]GnRH II for XGnRHRs) with or without an excess of homologous competitor to define total and non-specific binding, as described (9, 10). Receptor internalization was determined using a similar whole cell assay except that binding was measured (e.g. immunohistochemistry and ERK-GFP translocation) as indicated below. When investigated, we have found no effect of transfection method (Ad versus plasmid) on functional characteristics of transfected receptors or effectors (15) (data herein).
labeled by preincubation with [3H]inositol and stimulated in the presence of LiCl, as described (9, 10).

**Confocal Microscopy**—Cells were seeded onto glass coverslips and transfected with 2 μg of eDNA encoding either the 3F-hGnRHR or the HA-XGnRHR. After 24 h, cells were washed in DMEM containing no serum or antibiotics and then incubated for 1 h in DMEM containing either mouse monoclonal anti-HA (clone 11 at 1:200; Covance) or mouse monoclonal anti-FLAG (M2 at 1:200; Sigma). Medium was warmed to 37 °C, and cells were incubated for 15–30 min at 37 °C with or without 10−6 m buserelin (3F-hGnRHR) or GnRH II (HA-XGnRHR). Cells were then washed in ice-cold phosphate-buffered saline (PBS) and fixed in 2% paraformaldehyde, before permeabilization in PBS with 0.1% Triton X-100. Cells were incubated with Alexa-594 anti-mouse (1:500; Invitrogen) in PBS with 0.1% Triton X-100 and 1% bovine serum albumin prior to washing and mounting. Images were then captured using a Leica TCS-SP2 confocal laser-scanning microscope. Arrestin-GFP redistribution was assessed by live cell confocal microscopy as described (15), and similar methods were used for live cell imaging of ERK2-GFP. Briefly, cells were seeded onto glass coverslips and transfected with equal amounts of pERK2-GFP and pEXV3MEK1, with hGnRHR or XGnRHR in pCR3.1, as indicated. For titer-dependent assays, cells were transfected with pERK2-GFP and pEXV3MEK1 24 h prior to transfection with Ad hGnRHR or Ad XGnRHR at titers indicated in the figure legends. Cells were then starved in 0.1% FCS-DMEM for 16–24 h prior to stimulation and assay. Image capture was performed within 5 min of loading in 1 ml of phenol red-free HEPES-buffered DMEM containing 0.1% serum every 1–5 min as described above. For immunostaining of total and pp-ERK1/2, cells were seeded onto glass coverslips and transfected as above with Ad hGnRHR or Ad XGnRHR as indicated in the figure legends. For comparison with transfected cells, ERK2-GFP and MEK1 were transfected into cells prior to Ad transfection as outlined above. Cells were stimulated for the times indicated with 10−6 m buserelin (hGnRHR) or GnRH II (XGnRHR) prior to washing in ice-cold PBS, fixation in 10% paraformaldehyde, and permeabilization in methanol. Polyclonal rabbit anti-total ERK1/2 and rabbit anti-phospho-Thr202/Tyr204 ERK1/2 (1:250; Cell Signaling, Hitchin, UK) were used to stain cells in conjunction with Alexa-568-conjugated goat anti-rabbit secondary antibodies (1:250; Invitrogen).

**Measurement of ERK1/2 Activation by Western Blotting**—Activation of ERK1/2 was measured by Western blotting as described (9), using a modified extraction buffer consisting of 10 mM Tris, pH 7.6, 5 mM EDTA, 50 mM NaCl, 30 mM sodium pyrophosphate, 50 mM NaF, 1 mM dithiothreitol, 100 μM sodium orthovanadate, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitor mixture tablets (used according to the manufacturer’s instructions; Roche Applied Science). Total and pp-ERK1/2 were detected using polyclonal rabbit anti-total ERK1/2 and rabbit anti-phospho-Thr202/Tyr204 ERK1/2 (1:1000; Cell Signaling) on duplicate membranes and visualized by enhanced chemiluminescence. Bands were scanned and quantified by densitometry, and the pp-ERK1/2 was expressed as a percentage of maximal ERK phosphorylation.

**Statistical Analysis and Data Presentation**—The figures show the mean ± S.E. of data pooled from n independent experiments (raw data or data normalized as described in the legends of Figs. 2–4 and 8) or from the number of individual cells indicated in the legends of Figs. 6, 7, 9 and 10. The confocal microscopy images shown are representative of those obtained in at least three separate experiments. Where nuclear/cytoplasmic ratios of fluorophores were calculated, areas of interest were defined manually, and mean fluorescent intensity in these areas was determined using LCSLite software (Leica, Heidelberg, Germany).

Data are typically reported here as mean ± S.E., and statistical analysis was by Student’s t test, accepting p < 0.05 as statistically significant.

**RESULTS**

In the first experiments, rates and arrestin dependence of receptor internalization were assessed using whole cell radioligand binding assays with HeLa cells transfected with hGnRHRs or XGnRHRs. As expected, the XGnRHR was internalized faster than the hGnRHR (Fig. 1, upper panel; see also Refs. 9, 10, 14, and 31). Co-expression of arrestin-3-GFP had little effect on internalization of either receptor at 30 min, and co-expression of the dominant negative truncated arrestin, Δarrestin (319–418), reduced XGnRHR internalization by around 50% without altering internalization of the hGnRHR. When HA-tagged XGnRHRs were expressed in HeLa cells, they were located primarily at the plasma membrane in unstimulated cells, but after 30 min of stimulation with GnRH II (10−6 m), numerous XGnRHR-containing vesicles were observed within the cytoplasm (Fig. 1, lower panel, b and e). In additional experiments, internalized XGnRHRs were often co-localized with fluorescently labeled transferrin (Alexa-594-transferrin; not shown). Activation of these receptors also caused translocation of co-
expressed arrestin-3-GFP to the plasma membrane within minutes of stimulation (not shown) and then to vesicles within the cytoplasm (Fig. 1, lower panel a and d), where the arrestin was typically co-localized with XGnRHRs (Fig. 1, lower panel c and f). In unstimulated cells, FLAG-tagged hGnRHRs were observed both at the plasma membrane and with a punctate distribution in the cytoplasm (not shown). Activation of these receptors with 10^{-6} M buserelin (an agonist selective for type I mammalian GnRHRs) did not increase the number of receptor-containing vesicles (not shown). It also failed to influence arrestin-3-GFP distribution, and hGnRHR-containing vesicles were not seen co-localized with transferrin or with arrestin-3-GFP (not shown).

The data above suggest that the XGnRHR undergoes rapid agonist-induced and arrestin-dependent internalization via clathrin-coated vesicles, with consequent co-localization of XGnRHRs and arrestins in endosomes, as opposed to the slow and arrestin-independent internalization of the hGnRHR. Since the binding of some 7TM receptors to arrestin can mediate sustained activation of ERK, we compared hGnRHR- and XGnRHR-mediated ERK activation.

Amplitudes and kinetics, reaching maxima at 5 min and reducing thereafter to nearly basal levels at 60 min (Fig. 2, A and B). In other models, type I mammalian GnRHRs can act via G_{q/11} to stimulate a PKC- and/or EGF receptor-mediated activation of ERK. We therefore assessed the effects of a range of pharmacological inhibitors in Ad hGnRHR-infected HeLa cells stimulated with buserelin, PDBu, or EGF. An inhibitor of EGF receptor autophosphorylation (AG 1478) caused a dose-dependent inhibition of the EGF-stimulated ERK phosphorylation (IC_{50} < 30 nM; not shown), and a maximally effective concentration (100 nM) abolished the effect of EGF but had no effect on PDBu- or buserelin-stimulated ERK phosphorylation (Fig. 2C). The PKC inhibitor Ro31-8425 caused a dose-dependent inhibition of the response to PDBu (IC_{50} ~ 30 nM; not shown), and a maximally effective concentration (200 nM) abolished the response to PDBu but caused only partial inhibition of the hGnRHR-mediated response (~40%) and did not inhibit the response to EGF (Fig. 2C). Similar experiments were performed with Ad XGnRHR-infected cells stimulated with GnRH II, and the data obtained were indistinguishable from those with Ad hGnRHR-infected cells (not shown). Moreover, responses to these stimuli were not reduced by the Src inhibitor SU6659 (100 nM) or the phosphatidylinositol 3-kinase inhibitor wortmannin (25 nM), whereas responses to all stimuli were reduced by the MEK inhibitor PD98059 (25 mM) (not shown). We also assessed the effects of PKC inhibition on the kinetics of hGnRHR- and XGnRHR-mediated ERK activation and observed a comparable inhibitory effect of Ro31-8425, irrespective of the receptor used or time point measured (Fig. 3). Thus, it appears that both GnRHRs mediate rapid and transient activation of ERK that is independent of Src, phosphatidylinositol 3-kinase, and EGF receptor trans-activation and that for both receptors, the effect has PKC-dependent and PKC-independent components with comparable kinetics.
We next assessed the ability of an active or dominant negative arrestin to influence GnRHR-mediated ERK phosphorylation and found that arrestin-3-GFP, and Δarrestin-(319–418) had no measurable effect on the increase in pp-ERK1/2 mediated by activation of hGnRHRs. There was a tendency for arrestin-3-GFP to reduce XGnRHR-mediated ERK phosphorylation (to 80 ± 10% of control) and for Δarrestin-(319–418) to increase this response (to 135 ± 20% of control). Although not statistically different from control values (p > 0.1), these values do differ from one another (p < 0.05) and are consistent with a modest attenuation of this response by arrestin-dependent rapid homologous desensitization of the XGnRHR. Suspecting that PKC-mediated ERK activation may have obscured arrestin dependence, similar experiments were performed in the presence of Ro31-8425. As shown, XGnRHR-mediated ERK phosphorylation was abolished by Δarrestin-(319–418) in the presence of Ro31-8425, (Fig. 4B), whereas hGnRHR-mediated ERK phosphorylation was unaltered by Δarrestin-(319–418) under these conditions (Fig. 4A). Similar data were obtained when arrestin-2-GFP was used (in place of arrestin-3-GFP) and when transfection was with recombinant Ad (expressing hGnRHR, XGnRHR, or Δarrestin-(319–418)) as opposed to the Superfect-mediated transfection used for the experiments shown in Fig. 4 (not shown).

The data above provide evidence for three distinct ERK activation mechanisms: first, the PKC-dependent activation seen with both receptors; second, the arrestin-mediated activation mediated by the XGnRHR; and third, a PKC- and arrestin-independent activation via the hGnRHR. Since ERK activation mechanisms can have important consequences for its compartmentalization, we next used immunohistochemistry to determine the distribution of ERK and pp-ERK within the cell. Before stimulation, Ad hGnRHR-infected cells showed very little staining for pp-ERK1/2 (Fig. 5). Stimulation with buserelin caused a rapid and transient increase in pp-ERK1/2 staining with similar kinetics to that seen by Western blotting for pp-ERK1/2 (Figs. 2, 3, and 5 and data not shown). The hGnRHR-mediated increase in pp-ERK1/2 was markedly attenuated by Ro31-8425 but was not altered by Δarrestin-(319–418) in the presence of the PKC inhibitor (Fig. 5). The XGnRHR caused a similar transient and PKC-dependent increase in pp-ERK1/2, but the Ro31-8425-resistant increase in pp-ERK1/2 was almost entirely prevented by Δarrestin-(319–418), confirming the receptor-specific arrestin dependence seen earlier by Western blotting (Figs. 3 and 5). In control experiments, Δarrestin-(319–418) did not influence the increase in pp-ERK1/2 caused by 10−7 M PDBu (measured by Western blotting) or the increase in nuclear pp-ERK1/2 caused by 10−7 M PDBu (measured by immunohistochemistry). Examination of subcellular localization reveals that both GnRHRs increase pp-ERK1/2 within the nucleus and cytoplasm and that, for both receptors, the increase in nuclear pp-ERK1/2 is largely prevented by PKC inhibition (Fig. 5). Although there was too little staining for accurate assessment of the
ratio of nuclear/cytoplasmic pp-ERK1/2 in control cells, the values seen after stimulation of XGnRHRs and hGnRHRs were indistinguishable (1.71 ± 0.20 and 1.84 ± 0.05, respectively; n = 20 – 24 cells), and these were significantly reduced by Ro31-8425 (0.84 ± 0.20 and 1.84 ± 0.05, respectively; n = 25 – 29 cells).

Although none of these treatments altered the levels of total ERK in whole cell lysates or the extent of immunostaining, its distribution was altered by both receptors. Thus, ERK1/2 staining in quiescent cells was largely restricted to the cytoplasm, whereas stimulated cells showed rapid and transient accumulation of ERK1/2 in the nucleus, consistent with its translocation to the nucleus and a consequent net increase in nuclear ERK1/2 (not shown). In order to monitor this effect in more detail (and in live cells), we used an ERK2-GFP fusion protein that was transiently transected into the cells and visualized by confocal microscopy. When cells were transfected with ERK2-GFP and incubated in medium with 0.1% FCS, the fusion protein was evenly distributed between the nucleus and cytoplasm. This differs from the largely cytoplasmic distribution of endogenous ERK1/2 (above) and presumably reflects the excess of exogenous ERK over its cytoplasmic binding partner, MEK. Consequently, when ERK2-GFP is co-transfected with MEK1, the proportion of fusion protein in the cytoplasm is increased, recapitulating the distribution pattern of endogenous ERK1/2. In serum-starved cells co-transfected with ERK2-GFP and MEK1, EGFr and PDBu both cause a rapid translocation of ERK to the nucleus (i.e. increased the nuclear/cytoplasmic ERK2-GFP ratio) with differential kinetics, consistent with differences in ERK phosphorylation profiles, confirming the utility of this model for monitoring agonist-induced ERK2-GFP trafficking (Fig. 6; see also Refs. 20, 32, and 33). As further validation of the model, Western blotting revealed identical kinetics for phosphorylation of endogenous ERK1/2 and transfected ERK2-GFP, after stimulation with EGF or PDBu and after activation of transfected hGnRHRs and XGnRHRs (not shown). We also noted that expression levels of ERK2-GFP varied considerably from cell to cell and were concerned that this might influence the observed translocation response, but single cell analysis argues against this possibility. When cytoplasmic fluorescence was plotted against nuclear fluorescence (for ~70 individual cells before or after stimulation with EGF), two distinct cell populations were revealed. Less than 10% of unstimulated cells had higher fluorescence levels in the nucleus than the cytoplasm, whereas more than 90% of stimulated cells had higher fluorescence levels in the nucleus, and this distinction was observed over a wide range (at least 100-fold difference) of fluorescence intensities (not shown).

We next used this strategy to assess effects of GnRHR activation in cells that were transfected with ERK2-GFP and MEK1 along with hGnRHR or XGnRHR and then maintained in 0.1% FCS medium for 16 – 24 h prior to stimulation with 10−7 M buserelin or GnRH II. As shown (Fig. 7), the hGnRHR mediated no significant increase in nuclear/cytoplasmic ERK2-GFP over the 120-min stimulation period, whereas the XGnRHR mediated a rapid and transient increase, peaking only 5 min after stimulation. The fact that the hGnRHR increased pp-ERK1/2 within the nucleus (above) but did not increase the nuclear/cytoplasmic ratio of ERK-GFP underlines the differential scaffolding (and therefore different compartmentalization) of total and phosphorylated ERK.

In the experiments shown in Fig. 7, receptor number was not measured, but parallel experiments revealed ~20,000–30,000 XGnRHR/cell as compared with ~15,000 sites/cell for the hGnRHR (assuming transfection efficiency of 20%; data not shown). To address the relevance of receptor number more directly, we infected cells with Ad GnRHRs at a range of titers prior to quantification of receptor number and receptor-mediated effects on pp-ERK1/2, [3H]IP accumulation, and ERK2-GFP distribution. As shown (Fig. 8), increasing Ad hGnRHR titer (from 0 to 3 × 104 pfu/ml) increased receptor number from 0 to ~25,000 sites/cell, and increasing Ad XGnRHR titer (from 0 to 1 × 107 pfu/ml) increased receptor number from 0 to ~25,000 sites/cell. In both cases, this was associated with titer-dependent increases in receptor-mediated ERK phosphorylation and [3H]IP accumulation. A plot of receptor number against these responses reveals their expected dependence on receptor number, but both receptor-response curves were right-shifted for the XGnRHR (as compared with the hGnRHR). Thus, maximal hGnRHR-mediated ERK phosphorylation was achieved with less than 10,000 receptors/cell as compared with ~25,000 receptors/cell for the XGnRHR. Using this data set, we selected Ad titers giving comparable high
or low levels of receptor expression for comparison of ERK2-GFP translocation responses. When hGnRHRs and XGnRHRs were expressed at 20,000–25,000 receptors/cell (Ad titers of 3 × 10⁷ pfu/ml for the hGnRHR and 3 × 10⁶ pfu/ml for the XGnRHR), both receptors caused pronounced and transient ERK2-GFP translocation (Fig. 9A). In contrast, when the receptors were expressed at 7500–10,000 sites/cell (Ad titers of 1 × 10⁶ pfu/ml for the hGnRHR and 3 × 10⁵ pfu/ml for the XGnRHR), the XGnRHR mediated a pronounced and transient translocation of ERK2-GFP, whereas hGnRHR activation caused little net movement of kinase (Fig. 9B). Accordingly, the XGnRHR is more efficient than the hGnRHR at causing ERK translocation to the nucleus (Fig. 9) despite the fact that it is less efficient at increasing whole cell pp-ERK1/2 (as measured by Western blotting or immunohistochemistry) (Figs. 2–5), and this effect is partially dependent upon PKC (30–70% inhibited by Ro31-8425) but independent of EGF receptor trans-activation (uninfluenced by AG 1478) and also apparently independent of Src and phosphatidylinositol 3-kinase (uninfluenced by SU6659 or wortmannin).

In a final series of experiments, we determined the contribution of arrestin-mediated and PKC-mediated signaling to ERK2-GFP translocation. As shown (Fig. 10), activation of the XGnRHR caused the expected increase in nuclear/cytoplasmic ERK2-GFP. This translocation was increased by Δarrestin-(319–418) and inhibited by Ro31-8425 (in the presence or absence of Δarrestin-(319–418)), consistent with the earlier data demonstrating that nuclear accumulation of pp-ERK1/2 is dependent upon PKC activation (Fig. 5) and the ability of PKC to mediate ERK translocation to the nucleus in other systems (29). In control experiments, Δarrestin-(319–418) did not influence the nuclear translocation of ERK2-GFP stimulated by 10⁻⁷ M PDBu (not shown).

**DISCUSSION**

Phylogenetic analysis reveals that the GnRH receptor family has undergone a period of rapidly accelerated molecular evolution in which the advent of mammals is associated with the loss of the C-terminal tail (3). This is particularly remarkable in light of the presence of C-tails in all other 7TM receptors (and the wide range of functions attributed to these structures) and provides a unique opportunity for comparative studies to determine the roles of C-tails within normal (nonmutated) 7TM receptors. Here we show that upon activation, XGnRHRs are rapidly internalized in an arrestin-dependent manner, that they provoke translocation of arrestin (first to the plasma membrane and then to vesicles), and that they are co-localized (after stimulation) with transferrin and arrestin in vesicles. In contrast, hGnRHRs are slowly internalized in an arrestin-independent manner and are not seen co-localized with transferrin or arrestin in vesicles. These data suggest that the C-tailed XGnRHRs undergo rapid agonist-induced and arrestin-dependent internalization via arrestin-containing clathrin-coated vesicles, as opposed to the slow and arrestin-independent internalization of tail-less GnRHRs. They are consistent with earlier studies showing that hGnRHRs do not rapidly desensitize, whereas XGnRHRs do (9), as well as comparative studies with other GnRHRs (notably rat and catfish) demonstrating the importance of C-tails for agonist-induced phosphorylation, arrestin binding, desensitization, and internalization (4, 7–13, 34–36).

7TM receptors can be grouped according to their affinity for arrestins (37), and our data suggest that the XGnRHR is a group B receptor (having high affinity for arrestins and therefore co-localized with arrestin after internalization), whereas the hGnRHR is neither group A nor B (having essentially no affinity for arrestins). This distinction has important consequences for receptor function, since the high affinity of group B receptors for arrestins can favor signaling via arrestin-scaffolded effectors, including ERK (24, 26, 27, 38, 39). This raises the possibility that the differential propensity for arrestin binding by mammalian and nonmammalian GnRHRs might influence the efficiency and/or cellular compartmentalization of ERK activation. Although the mechanisms by which nonmammalian GnRHRs activate MAP kinases have not been extensively explored, type I mammalian GnRHRs are known to act via G₂₁ to stimulate the ERK, c-Jun N-terminal kinase, and p38 MAPK cassettes (40), and the mechanisms by which they activate ERK differ between cell types. Thus, the endogenous mouse GnRHRs of gonadotrope lineage αT₃-1 cells act primarily via activation of PKC and entry of Ca²⁺ to cause a Raf-1-dependent activation of ERK (40, 41). In these cells, EGF receptor trans-activation is not required (42, 43), whereas ERK activation by mouse GnRHRs stably transfected into COS7 cells is partially dependent upon PKC (30–70% inhibited by Ro31-8425) but independent of EGF receptor trans-activation (uninfluenced by AG 1478) and also apparently independent of Src and phosphatidylinositol 3-kinase (uninfluenced by SU6659 or wortmannin).
Activation of some 7TM receptors (e.g. AT1ARs) causes a sustained increase in ERK phosphorylation that reflects a rapid and transient PKC-mediated activation followed by a slower but more sustained arrestin-mediated activation (45), which is apparently associated with the group B profile of sustained, ubiquitin-dependent association of arrestin and receptor (22, 27, 39, 45). However, this temporal uncoupling is not always seen, because, for example, PAR2 and NK1R activation can cause activation of ERK that is blocked by the dominant negative arrestin-(319–418) during both initial and sustained phases of the response, despite indications by immunostaining that both are class B receptors (24, 38, 46). Here, we show that both hGnRHRs and XGnRHRs mediate a rapid and transient activation of ERK and that the magnitude and kinetics of responses to these two receptors are indistinguishable (Fig. 2). Moreover, PKC inhibition was effective at all time points, such that time courses were comparable in the presence and absence of Ro31-8425 for both receptors (Fig. 3). Nevertheless, the PKC-independent activation of ERK by the XGnRHR was abolished by arrestin-(319–418), whereas the PKC-independent activation of ERK by the hGnRHR was uninfluenced by the dominant negative arrestin (as measured by Western blotting or immunohistochemistry for pp-ERK1/2) (Figs. 4 and 5). These observations are in accord with earlier studies demonstrating no role for arrestins in ERK phosphorylation mediated by rat GnRHRs (42) and are consistent with the fact that rat and human GnRHRs do not bind arrestin (15, 34). In contrast, the inhibitory effect of arrestin-(319–418) on XGnRHR-mediated ERK phosphorylation most likely reflects blockade of the scaffolding function of endogenous arrestins and, as such, provides the first direct evidence for arrestin-mediated signaling of a GnRHR. In this context, the comparable kinetics of XGnRHR-mediated ERK phosphorylation most likely reflects blockade of the scaffolding function of endogenous arrestins and, as such, provides the first direct evidence for arrestin-mediated signaling of a GnRHR. This is presumably why the arrestin dependence was observed only after PKC inhibition, a procedure expected to remove the confounding effect of arrestin-dependent receptor desensitization on XGnRHR-mediated ERK activation and to increase the signal/noise ratio for the residual arrestin-mediated component of ERK activation. It is also important to recognize that phosphatase activity may oppose sustained ERK activation and that GnRHR activation has been shown to induce MAPK-phosphatase 1 and 2 (47), this effect could prevent sustained ERK activation and thereby prevent temporal uncoupling of responses mediated by PKC and arrestins.
The relationship between ERK activation and distribution is complex, with potential for regulation by phosphorylation and dephosphorylation (within the cytoplasm and/or nucleus) as well as binding to scaffolds or chaperones (again, within the cytoplasm and/or nucleus). It is increasingly evident that stimuli causing comparable whole cell ERK activation responses may have different effects on its compartmentalization. Since PKC activation typically causes ERK translocation to the nucleus, many G_{q/11}-coupled 7TMs also provoke nuclear translocation. Nevertheless, scaffolding of ERK to arrestin bound to class B 7TMs can retain pools of ERK in the cytoplasm, often associated with endosomes (24-27), and we therefore suspected that XGnRHR activation would cause such retention. We found that XGnRHR activation caused a pronounced increase in pp-ERK1/2 in both the nucleus and the cytoplasm but that the increase in nuclear pp-ERK1/2 was markedly attenuated by Ro31-8425. Thus, PKC-mediated ERK activation causes nuclear accumulation of pp-ERK1/2, whereas the arrestin-mediated activation apparently causes cytoplasmic retention (Fig. 5). The transient nature of the responses seen in the presence of Ro31-8425 (Fig. 3) indicates the potential involvement of cytoplasmic phosphatases in addition to nuclear MAPK phosphatase 1 and 2 (47) in determining the kinetics of the response to GnRHRs. An additional unexpected finding was that ERK activation by hGnRHRs was only partially dependent upon PKC. The mechanisms underlying this PKC-independent ERK activation by the hGnRHR are unknown (the effect is defined only by lack of dependence on PKC and arrestin) but could conceivably be related to scaffolding within focal adhesions, because the rat GnRHR has been shown to activate ERK by a mechanism dependent upon assembly of ERK and focal adhesion kinase within focal adhesions (48).

In addition to monitoring the subcellular distribution of pp-ERK1/2, we established an ERK2-GFP translocation model to track movement of the kinase in live cells. In our initial studies, we were surprised to find that the XGnRHR mediated a pronounced and transient increase in the net movement of ERK2-GFP to the nucleus (Fig. 6), whereas the hGnRHR caused no such effect. However, in a larger series of experiments in which Ad titer was varied, we observed the expected positive relationship between receptor number, [*H]IP$_x$ accumulation (rate of phospholipase C activation), and ERK phosphorylation and that the GnRHRs (like the XGnRHRs) are capable of mediating ERK2-GFP translocation to the nucleus when sufficient receptors are expressed (Figs. 8 and 9). In interpreting these data, it is important to recognize that the minimal Ad titer used (10$^6$ pfu/ml) represents a multiplicity of infection (the ratio of Ad particles to infected cells) of $\sim$10, and we have shown that over 90% of HeLa cells are transfected at this multiplicity of infection (10). Accordingly, the titer-dependent increase in binding reflects an increase in receptors per cell (rather than the proportion of cells transfected). Moreover, the receptor expression levels achieved are relatively low (maximally 25,000 sites/cell for the hGnRHR, as compared with 20,000–80,000 sites/cell for endogenous type I mammalian GnRHRs in gonadotropes (9)), whereas the levels of receptor occupancy are relatively high (10$^{-6}$ M buserelin or GnRH II would be expected to bind 100% of the cognate receptor, whereas physiological concentrations of GnRH I occupy <50% of GnRHRs in vivo). Indeed, these experiments were designed to combine low receptor number and high percentage occupancy in order to approximate physiological levels of type I GnRHR occupancy in gonadotropes. Using these internally controlled data, we were able to compare ERK2-GFP translocation under conditions matched for receptor number and to determine the relationship between receptor number and ERK2-GFP translocation, a relationship that (to our knowledge) has not been explored for any other receptor.

We find that the dose-response curves for XGnRHR-mediated [*H]IP$_x$ accumulation and ERK phosphorylation are right-shifted as compared with the corresponding curves for the hGnRHR (Fig. 8; see also Refs. 9 and 10) and that under conditions matched for high receptor number (and occupancy), both receptors mediate comparable rapid and transient increases in cytoplasmic/nuclear ERK2-GFP ratio (Fig. 9). However, under conditions matched for low receptor number (7500–10,000 sites/cell), the XGnRHR mediated net nuclear translocation, whereas the hGnRHR did not (Fig. 9).

In the final series of experiments (Fig. 10), we assessed the dependence of XGnRHR-mediated ERK translocation on PKC and arrestin and found that translocation was increased by Jarrestin (319–418). This is consistent with the scaffolding of ERK to arrestin and consequent inhibition of translocation, but could also reflect inhibition of XGnRHR desensitization and a consequent increase in ERK activation (the XGnRHR-mediated increase in pp-ERK1/2 was 135±25% of control in the presence of Jarrestin (319–418), and, although not statistically significant, this increment is comparable with that seen for ERK2-GFP translocation). The XGnRHR-mediated translocation of ERK2-GFP was also inhibited by Ro31-8425, consistent with the possibility that PKC activation increases translocation of pp-ERK1/2 to the nucleus, with a consequent increase in total ERK within the nucleus. In light of this, it is remarkable that ERK translocation is seen upon activation of 7500–10,000 XGnRHRs (with which no measurable stimulation of [*H]IP$_x$ accumulation occurs) but not with activation of the same number of hGnRHRs (with which clear stimulation of [*H]IP$_x$ accumulation occurs). Accordingly, the XGnRHR is more efficient than the hGnRHR at causing ERK translocation to the nucleus (Fig. 9) despite the fact that it is less efficient at increasing whole cell pp-ERK1/2 (Fig. 8). The relatively inefficient hGnRHR-mediated ERK2-GFP translocation revealed at low receptor number implies that mechanisms other than arrestin scaffolding can hinder ERK translocation in these cells. As noted above, these could include ERK scaffolding within focal adhesions (48).

Overall, these data are consistent with the possibility that the molecular evolution of GnRHRs (e.g. the advent of tailless mammalian GnRHRs) has been associated with a major change in signaling repertoire, namely the loss of arrestin-dependent (probably G-protein-independent) signaling. In this context, we have found that the XGnRHR signals to ERK via both PKC and arrestin, whereas the hGnRHR signals to ERK via PKC and an additional pathway defined only by its lack of dependence on PKC, arrestin, EGF receptor activation, Src, and phosphatidylinositol 3-kinase. Our data provide the first direct evidence for arrestin-mediated GnRHR signaling and suggest that differences in ERK scaffolding may influence the efficiency with which these distinct pathways provoke nuclear translocation. The PKC-mediated phosphorylation of ERK is clearly associated with nuclear translocation, whereas the arrestin-mediated activation may cause cytoplasmic retention (seen only when the PKC-dependent translocation is prevented). The PKC- and arrestin-independent pathway activated by hGnRHRs (but not by XGnRHRs) also appears to cause cytoplasmic retention, explaining the relative inefficiency of hGnRHRs at causing translocation and suggesting a role for scaffolds other than arrestin in signaling of mammalian GnRHRs to ERK. In this context, it is important to note that the hGnRHR mediated nearly maximal ERK phosphorylation with very little XGnRHR-mediated increase in pp-ERK1/2 was 135±25% of control in the presence of Jarrestin (319–418), and, although not statistically significant, this increment is comparable with that seen for ERK2-GFP translocation). The XGnRHR-mediated translocation of ERK2-GFP was also inhibited by Ro31-8425, consistent with the possibility that PKC activation increases translocation of pp-ERK1/2 to the nucleus, with a consequent increase in total ERK within the nucleus. In light of this, it is remarkable that ERK translocation is seen upon activation of 7500–10,000 XGnRHRs (with which no measurable stimulation of [*H]IP$_x$ accumulation occurs) but not with activation of the same number of hGnRHRs (with which clear stimulation of [*H]IP$_x$ accumulation occurs). Accordingly, the XGnRHR is more efficient than the hGnRHR at causing ERK translocation to the nucleus (Fig. 9) despite the fact that it is less efficient at increasing whole cell pp-ERK1/2 (Fig. 8). The relatively inefficient hGnRHR-mediated ERK2-GFP translocation revealed at low receptor number implies that mechanisms other than arrestin scaffolding can hinder ERK translocation in these cells. As noted above, these could include ERK scaffolding within focal adhesions (48).

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