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Differential Internalization of Mammalian and Non-mammalian Gonadotropin-releasing Hormone Receptors

UNCOUPLING OF DYNAMIN-DEPENDENT INTERNALIZATION FROM MITOGEN-ACTIVATED PROTEIN KINASE SIGNALING*

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Desensitization and internalization of G-protein-coupled receptors can reflect receptor phosphorylation-dependent binding of β-arrestin, which prevents G-protein activation and targets receptors for internalization via clathrin-coated vesicles. These can be pinched off by a dynamin collar, and proteins controlling receptor internalization can also mediate mitogen-activated protein kinase signaling. Gonadotropin-releasing hormone (GnRH) stimulates internalization of its receptors via clathrin-coated vesicles. Mammalian GnRH receptors (GnRH-Rs) are unique in that they lack C-terminal tails and do not rapidly desensitize, whereas non-mammalian GnRH-R have C-terminal tails and, where investigated, do rapidly desensitize and internalize. Using recombinant adenovirus expressing human and *Xenopus* GnRH-Rs we have explored the relationship between receptor internalization and mitogen-activated protein kinase signaling in HeLa cells with regulated tetracycline-controlled expression of wild-type or a dominant negative mutant (K44A) of dynamin. These receptors were phospholipase C-coupled and had appropriate ligand affinity and specificity. K44A dynamin expression did not alter human GnRH-R internalization but dramatically reduced internalization of *Xenopus* GnRH-R (and epidermal growth factor (EGF) receptor). Blockade of clathrin-mediated internalization (sucrose) abolished internalization of all three receptors. Both GnRH-Rs also mediated phosphorylation of ERK 2 and for both receptors, this was inhibited by K44A dynamin. The same was true for EGF- and protein kinase C-mediated ERK 2 phosphorylation. ERK 2 phosphorylation was also inhibited by a protein kinase C inhibitor but not affected by an EGF receptor tyrosine kinase inhibitor. We conclude that a) desensitizing and non-desensitizing GnRH-Rs are targeted for clathrin-coated vesicle-mediated internalization by functionally distinct mechanisms, b) GnRH-R signaling to ERK 2 is dynamin-dependent and c) this does not reflect a dependence on dynamin-dependent GnRH-R internalization.

Sustained stimulation of most G-protein-coupled receptors (GPCRs)[1] causes their desensitization and internalization from the cell surface. For many GPCRs this is mediated by G-protein receptor kinases, which phosphorylate the receptor and promote the binding of β-arrestin and a consequent reduction in coupling of the receptor to its heterotrimeric G-proteins (1). β-arrestin also serves as an adapter, targeting the desensitized receptor to clathrin-coated vesicles (CCVs) for internalization (2). Once formed, the CCV is "pinched" off from the plasma membrane by an oligomeric dynamin "collar". This effect of dynamin is dependent upon its intrinsic GTPase and can be blocked by expression of GTPase-inactive (dominant negative) mutants such as K44A dynamin (3). The internalized receptors are then either recycled back to the surface membrane or targeted to lysosomes for degradation so that agonist-induced GPCR internalization can reinforce desensitization or underlie resensitization.

Although this general scheme appears applicable to numerous GPCRs, there are important exceptions. For example, internalization of the m2 muscarinic acetylcholine receptor and the angiotensin 1A receptor is independent of both β-arrestin and dynamin (4–5) and internalization of endothelin 1A receptors is apparently independent of both β-arrestin and clathrin (6–7). Thus, although these issues remain controversial (see Refs. 8–11) for different GPCRs (and perhaps for different cell types), the adapter protein targeting receptors for internalization need not be β-arrestin, the fission protein regulating internalization need not be dynamin, and the vesicle need not be clathrin-coated.

Recent studies suggest that proteins mediating GPCR internalization may also mediate signaling. Thus β-arrestin can apparently recruit Src to agonist-occupied receptors, thereby facilitating Ras-dependent extracellular signal-regulated kinase (ERK) activation (12), so that the receptor, which is desensitized in terms of G-protein-mediated signaling may actually be activated in terms of G-protein-independent signaling. In addition, dominant negative forms of dynamin can inhibit

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[1] The abbreviations used are: GPCR(s), G-protein-coupled receptor(s); CCV(s), clathrin coated vesicle(s); ERK, extracellular signal-regulated kinase; MAP, mitogen-activated protein; EGF, epidermal growth factor; GnRH-R(s), gonadotropin-releasing hormone receptor(s); Ad, adenovirus(es); hGnRH-R(s), human GnRH-R(s); XGnRH-R(s), *Xenopus laevis* type I GnRH-R(s); PLC, phosphoinositide-specific phospholipase C; PKC, protein kinase C; cGnRH, chicken GnRH; wt, wild-type; IP, inositol phosphate; m.o.i., multiplicity of infection; PSS, physiological saline solution; tet, tetracycline; PMA, phorbol 12-myristate 13-acetate; EGFP, enhanced green fluorescent protein; MEK, MAP/ERK kinase.

mitogen-activated protein (MAP) kinase activation by GPCRs (13, 14), an effect that could reflect a requirement of GPCR internalization for MAP kinase activation (15) or a requirement for dynamin-dependent internalization of downstream proteins such as MAP/ERK kinase (MEK) (16). Indeed, since transactivation of the epidermal growth factor (EGF) receptors (17–18) or release of EGF (19) can underlie GPCR-mediated MAP kinase activation, the dynamin dependence of this effect could also reflect the known dynamin dependence of tyrosine kinase receptor-mediated MAP kinase activation (3).

The gonadotropin-releasing hormone receptor (GnRH-R) is a phospholipase C-coupled GPCR that mediates neuroendocrine control of gonadotropin secretion from pituitary gonadotropes and thereby plays a central role in control of reproduction. GnRH-Rs have a number of atypical structural characteristics (20), notably the unique absence of C-terminal tails in type I mammalian GnRH-Rs. In contrast, all cloned non-mammalian GnRH-Rs possess such tails and, since the serine and threonine residues that are phosphorylated by GRKs are often found within the C-terminal tail, comparative studies have addressed the functional relevance of these structures. These have revealed that mammalian GnRH-Rs do not show rapid homologous desensitization (21–22), whereas non-mammalian GnRH-Rs do rapidly desensitize (23) and that, although mammalian GnRH-Rs undergo agonist-induced internalization via CCVs, they are internalized much more slowly than non-mammalian GnRH-Rs. Moreover, where investigated, non-mammalian GnRH-Rs show agonist-induced phosphorylation and β-arrestin translocation and undergo β-arrestin-dependent internalization (24), whereas mammalian GnRH-Rs do not (25). Using recombinant adenovirus expressing human and type I *Xenopus* GnRH-Rs (Ad hGnRH-R and Ad XGnRH-R) we have recently shown that these functional distinctions (desensitization and internalization) are retained when GnRH-Rs are expressed at physiological density and in gonadotrope lineage cells (26). This supports the notion that GnRH-Rs have undergone a relatively recent period of accelerated molecular evolution that is functionally relevant and pertinent to the development of mammalian reproductive strategies (27).

Here, we have explored the dynamin dependence of GnRH-R internalization and MAP kinase signaling using recombinant adenovirus to express human and *Xenopus* GnRH-Rs in HeLa cells conditionally expressing wild-type or dominant negative (K44A) dynamin under tetracycline control (tet-OFF). Both receptors were positively coupled to PLC and MAP kinase (ERK 2 phosphorylation) and, as anticipated, the non-mammalian GnRH-Rs rapidly desensitized and internalized, whereas these processes were slower or absent for the mammalian GnRH-Rs. Both receptors were internalized via CCVs (as indicated by sucrose dependence) but internalization of the XGnRH-R was primarily dynamin-dependent, whereas that of the hGnRH-R was not. MAP kinase activation was dynamin-dependent for both receptors, was reduced by inhibition of internalization or of PKC activity, and was not inhibited by an EGF receptor, tyrophostin. Thus, desensitizing and non-desensitizing GnRH-Rs undergo functionally distinct forms of internalization and show dynamin-dependent signaling that does not reflect internalization of the GPCR or EGF receptor transactivation.

EXPERIMENTAL PROCEDURES

Materials and Cell Culture—GnRH and chicken GnRH II (cGnRH-II) were purchased from Peninsula Laboratories Europe Ltd. (Mersyside, United Kingdom) or from Sigma. Buserelin and ^{125}I-buserelin (2000 Ci/mmol) were provided by Prof. Sandow (Aventis Pharma GmbH, Frankfurt, Germany). ^{125}I-cGnRH-II (~3400 Ci/mmol as determined by self-displacement) was prepared using chloramine T and purified by G25 Sephadex column chromatography.^{125}I-EGF was purchased from PerkinElmer Life Sciences. AG1478 was purchased from Calbiochem. Culture media, sera, and plasticware were from Life Technologies, Inc. or Falcon (Becton Dickinson, Oxford, UK). Lipofectin, LipofectAMINE, and plus reagent were from Life Technologies, Inc., and FuGENE 6 was from Roche Molecular Biochemicals. 2-[^{3}H]inositol (14–16 Ci/mmol) was from Amersham International (Little Chalfont, UK). Recombinant adenovirus expressing the human GnRH-R (Ad hGnRH-R), the *Xenopus* type I GnRH-R (Ad XGnRH-R) and EGFP (Ad EGFP) were generated as previously described (26), and CMV-EGFP was from CLONTECH. All other reagents were from standard commercial suppliers. HeLa cells stably expressing either the wild-type (WT dynamin cells) or the dominant negative GTPase-deficient mutant dynamin (K44A dynamin cells) (kindly provided by Dr. S. Schmidt, Scripps Institute, La Jolla, CA) were cultured in serum-supplemented Dulbecco's modified Eagle's medium with G418 (300 μg/ml), puromycin (100 ng/ml), and tetracycline (1 μg/ml) as described (3). For experiments cells were harvested by trypsinization, plated in Dulbecco's modified Eagle's medium supplemented with 2% serum, and incubated for 2 days in flasks or culture plates as described in figure legends. Cells were infected on the second day, and where appropriate the tetracycline was removed. After 8 h, the medium was replaced with fresh medium, with or without tetracycline.

Accumulation of [^{3}H]Inositol phosphates ([^{3}H]IP)—[^{3}H]IP accumulation was used as a measure of PLC activity as described (28) using cells labeled by pre-incubation with [^{3}H]inositol and stimulated in the presence of LiCl. Cells were cultured in 24-well plates in 1 ml of media, and 2 μCi 2-[^{3}H]inositol (14–16 Ci/mmol) was added to each well for the final 16 h of incubation. After two washes with PSS (127 mM NaCl, 1.8 mM $CaCl_2$, 5 mM KCl, 2 mM $MgCl_2$, 0.5 mM NaH_2PO_4, 5 mM $NaHCO_3$, 10 mM glucose, 0.1% (w/v), bovine serum albumin and 10 mM HEPES, pH 7.4), each well was stimulated for the period indicated in the figures with 200 μl of PSS containing 10 mM LiCl and the indicated concentration of buserelin, GnRH, or cGnRH-II. The stimulation was terminated by adding 1 ml of water at 95 °C. The cells were lysed by freezing and thawing and [^{3}H]IP was separated from [^{3}H]inositol using anion exchange chromatography in formate form Dowex-1 columns, and the amount of ^{3}H eluted in each fraction was determined by liquid scintillation spectroscopy (28).

Radioligand Binding and Internalization Assays—GnRH-R expression was assessed and competition curves were constructed using whole cell binding assays in which ~50,000 cells were incubated in suspension for 30 min at 21 °C in 100 μl of PSS containing 1 mg/ml bacitracin with ~10^{-10} M radiolabel and 0 or 10^{-10} to 10^{-5} M of the unlabeled competitor peptide (26). Free and bound peptide were then separated by centrifugation through oil. For the human GnRH-R, the radiolabel was ^{125}I-buserelin and for *Xenopus* GnRH-R the radiolabel was ^{125}I-cGnRH-II. Similar assays were performed to quantify cell surface EGF receptors. Approximately 50,000 cells were incubated in suspension for 3 h at 4 °C in 100 μl PSS containing 1 mg/ml bacitracin with ~0.2 nM ^{125}I-EGF and 0 or 10^{-6} M of unlabeled EGF. Free and bound peptides were then separated by centrifugation through oil.

Internalization of EGF receptors was quantified in a modified whole cell binding assay in which ~250,000 cells were grown in 6-well plates. Cells were washed in PSS and then incubated at 37 °C in 1 ml of PSS containing ~10^{-7} M EGF. After the required incubation period (2–45 min) the cells were rapidly rinsed in ice-cold PSS (two times) and then incubated for 5 min in ice-cold 150 mM NaCl,50 mM acetic acid (pH 3) before scraping, pelleting, and resuspending in PSS. Internalization of GnRH-R was quantified in a modified whole cell binding assay in which ~50,000 cells (grown in 24-well plates) were washed in PSS and then incubated at 37 °C in 200 μl of PSS containing ~10^{-10} M radiolabel and 0 (total binding) or 10^{-6} M (nonspecific binding) of buserelin or cGnRH-II. After the required incubation period (5–30 min) the cells were rapidly rinsed in ice-cold PSS (two times) and then incubated for 2 min either in ice-cold PSS or in ice-cold 150 mM NaCl,50 mM acetic acid (pH 3). The cells were then washed three more times in ice-cold PSS and solubilized in 0.5 ml of 0.2 M NaOH with 1% SDS. Radiolabel in the solubilized cells was determined by γ counting, and specific cell-associated radioactivity was determined by subtraction of nonspecific from the total. Total specific binding is defined as the specific binding in cells receiving no acid wash, whereas acid-resistant (internalized)-specific binding is defined as that seen in the acid washed cells. An internalization index was calculated by expressing acid-resistant-specific binding as a percentage of total cell-associated-specific binding.

Fluid phase endocytosis was determined by measuring uptake of horseradish peroxidase as described (29). Briefly, cells were cultured at 250,000 cells/well in 6-well plates and infected with Ad hGnRH-R or Ad XGnRH-R (m.o.i. 100) as above. They were then incubated for 60 min at

37 °C in 0.5 ml of Dulbecco's modified Eagle's medium containing 5 mg/ml horseradish peroxidase (Sigma) and 0 or 10^{-7} M GnRH or cGnRH-II. Uptake was terminated by repeated washing in ice-cold phosphate-buffered saline prior to cell lysis with 0.1% Triton X-100 in phosphate-buffered saline. Horseradish peroxidase activity was then determined using 3,3-diaminobenzidine as a substrate followed by colorimetric measurement at 450 nm.

Dynamic Video Imaging of Cystolic Ca^{2+}—Cytosolic Ca^{2+} concentration was measured by dynamic video imaging using MagiCal hardware and Tardis software with a Nikon Diaphot microscope with ×40 quartz oil immersion objective (30). Cells were cultured on glass coverslips in flat-bottomed 12-well plates and were washed and then incubated for 30 min at 37 °C with PSS containing 2 μM fura-2 acetoxmethyl ester (fura-2/AM). Immediately before imaging the coverslip was dipped into PSS, blotted, and mounted on a greased incubation chamber. The cells were kept immersed in media constantly. Media changes were carried out by pipetting 4 ml of solution into the chamber, while an aspiration tube removed excess liquid and maintained a constant volume of around 400 μl. The samples in the chamber were illuminated alternately at 340 and 380 nm, and the ratio of the light emitted at 510 nm was measured, averaging 16 video frames to minimize the pixel to pixel standard error. This ratio was proportional to the intracellular ionized Ca^{2+}. For calibration, a dissociation constant of 225 nM for fura-2 and Ca^{2+} at 37 °C was used, and minimal and maximal fluorescence at 340 and 380 nm were determined in cells loaded with dye and made permeable to extracellular Ca^{2+} with 10 μM ionomycin in solutions containing either 10 mM Ca^{2+} or 10 mM EGTA.

Measurement of ERK 2 Activation by Western Blotting—Activation of ERK 2 was measured by Western blotting according to standard techniques. Briefly HeLa cells were plated in 6-well plates at 250,000 cells/well, infected with either Ad XGnRH-R or Ad hGnRH-R at an m.o.i. of 50–100, and left to express for 24 h. Following treatment, the cells were washed twice in ice-cold phosphate-buffered saline before being lysed on ice for 10 min in 400 μl of extraction buffer (10 mM Tris, pH 7.6, 5 mM EDTA, 1 mM EGTA, 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, 10 μg/ml antipain, 2 μg/ml leupeptin, 2 μg/ml pepstatin). Samples were then centrifuged (13,000 × *g*, 4 °C, 10 min) to clear the supernatant before 40 μl of aliquots were boiled with 40 μl of sample buffer. Proteins were separated by SDS-polyacrylamide gel electrophoresis (8% gel), transferred to polyvinylidene difluoride membrane and blocked with 5% skimmed milk/Tris-buffered saline Tween. ERK 2 was detected using monoclonal anti-ERK 2 (Santa Cruz) and visualized using enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech). The unphosphorylated and phosphorylated forms of ERK 2 were distinguished by retardation of the latter in SDS-polyacrylamide gel electrophoresis. Both bands were scanned and quantified by densitometry (Quantity 1 Software, Bio-Rad), and the phosphorylated ERK 2 was expressed as a percentage of total ERK 2 (both bands).

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 figure legends). Data are typically reported in text as mean ± S.E., and statistical analysis was by analysis of variance and Student's *t* test, accepting $p < 0.05$ as statistically significant. EC_{50} values were estimated by non-linear regression using "Graphpad Prism" (Graphpad Prism, San Diego, CA).

RESULTS

To explore the dynamin dependence of GnRH-R desensitization, internalization, and signaling, we sought to express human and *Xenopus* GnRH-Rs in HeLa cells expressing wild-type or K44A dynamin under control of the tetracycline-response element and a tetracycline-controlled transactivator. However, transfection efficiencies were found to be extremely low in these cells when conventional strategies ($CaPO_4$, Lipofectin, LipofectAMINE Plus, and FuGENE) were used. Using flow cytometry to assess protein expression after transfection with a CMV-EGFP plasmid we estimated that <20% of cells were transfected. In contrast transfection efficiencies approaching 100% were obtained using recombinant EGFP-expressing adenovirus (not shown). We therefore made use of previously generated recombinant adenovirus expressing human and *Xenopus* laevis type I GnRH-Rs (Ad hGnRH-R and Ad XGnRH-R, respectively).

Since these receptors have not (to our knowledge) been previously expressed in HeLa cells, it was necessary to first perform a basic pharmacological characterization. This was undertaken using WT dynamin cells maintained in medium with tetracycline (conditions not permissive for transgene expression). To determine the relationship between viral titer and receptor expression we constructed competition binding curves using ^{125}I-buserelin and varied amounts of unlabeled buserelin. No specific binding was seen in untransfected cells, but infection with Ad hGnRH-R with increasing titer (from m.o.i. of 3–100) increased ^{125}I-buserelin binding, and this was inhibited in a concentration-dependent manner by unlabeled buserelin. Fitting this data to a single site competition model revealed no dependence of K_d value on viral titer, so B_{max} values were estimated by refitting the data with the K_d fixed at the mean value of 2.4 nM (2.4 nM ± 0.3 nM, $n = 3$). This revealed receptor densities of 3000, 14,600, 25,500, and 46,500 sites/cell at m.o.i. values of 3, 10, 30, and 100, respectively (Fig. 1). Similar experiments with ^{125}I-cGnRH-II and unlabeled cGnRH-II in cells infected with Ad XGnRH-R again revealed high affinity binding sites with no relationship between Ad titer and K_d. The data was therefore fitted through the mean K_d value of 3.1 nM (3.1 nM ± 0.9 nM, $n = 3$). This revealed receptor densities of 6600, 17,000, 49,000, and 215,000 sites/cell at m.o.i. values of 3, 10, 30, and 100, respectively (data not shown).

To establish whether these binding sites were functional GnRH-Rs, GnRH-stimulated [^{3}H]IP accumulation was measured in cells infected with Ad hGnRH-R at varied titer. No stimulation was seen in untransfected cells or in cells infected at low viral titer (m.o.i. of 3), but GnRH did cause a clear dose- and titer-dependent stimulation of [^{3}H]IP accumulation at higher titers. At m.o.i. values of 3, 10, 30, and 100, 4.7% ± 2.0%, 3.8% ± 0.9%, 11.8% ± 3.3, and 31.7% ± 7.5%, respectively, of total [^{3}H]inositol was found in the [^{3}H]IP fraction after 30 min stimulation with a maximally effective concentration of GnRH (Fig. 1). A similar dose- and titer-dependent stimulation of [^{3}H]IP accumulation was seen in HeLa cells infected with Ad XGnRH-R (not shown). When these cells were stimulated for 30 min with 10^{-6} M cGnRH-II after infection at m.o.i. values of 3, 10, 30, and 100, cGnRH increased the proportion of [^{3}H]inositol recovered in the [^{3}H]IP fraction to 3.6% ± 0.2%, 5.9% ± 0.9%, 13.1% ± 4.1, and 32.3% ± 1.7%, respectively. Interestingly, the maximal observed [^{3}H]IP responses were comparable (~32%) for both receptors despite the fact that 4–5 times more XGnRH-R were expressed at the m.o.i. of 100. This distinction is similar to that observed with the same GnRH-R expressed in αT4 pituitary cells and may reflect differential desensitization during the course of the [^{3}H]IP accumulation assay (26).

Since activation of PLC mediates elevation of $[Ca^{2+}]_i$ by GnRH in pituitary cells, we also assessed possible effects of GnRH on Ca^{2+} in Ad hGnRH-R-infected HeLa cells. As shown (Fig. 1), GnRH did not increase $[Ca^{2+}]_i$ in control fura-2-loaded HeLa cells but caused a sustained and titer-dependent increase after infection at m.o.i. values of 3–100. Since these data were acquired by video imaging it was also possible to determine the proportion of cells responding to GnRH. This revealed that the vast majority of cells (>80%) responded to GnRH at relatively low titer (m.o.i. values of 10–30), and that increasing viral titer above an m.o.i. of 10 increased the amplitude of the responses without increasing the proportion of cells responding. These data (not shown) are very similar to those previously obtained when Ad GnRH-R were used to infect pituitary cells (26).

To determine the ligand specificity of GnRH-Rs expressed in these cells competition binding assays were performed using unlabeled GnRH, buserelin, and cGnRH-II and ^{125}I-buserelin

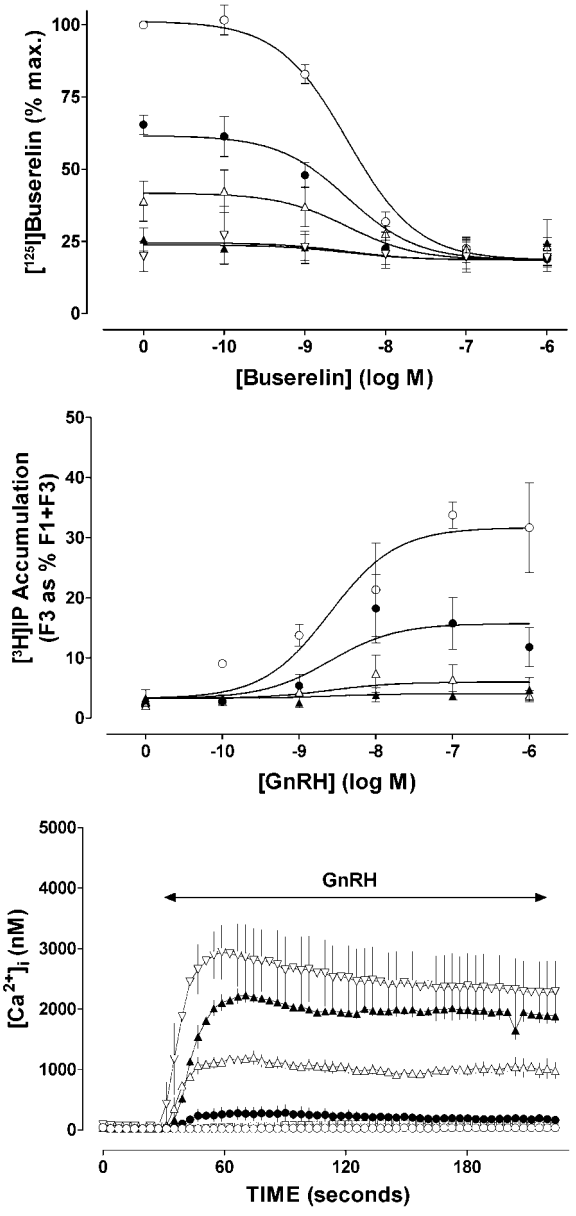

FIG. 1. **Titer dependence of receptor expression and signaling in WT dynamin cells infected with Ad hGnRH-R.** *Upper panel,* WT dynamin cells were cultured in 60-mm Petri dishes in the presence of tetracycline and infected with Ad hGnRH-R at m.o.i. values of 100 (○), 30 (●), 10 (△), 3 (▲), and 0 (▽) and then cultured for a further 24 h before being scraped from the culture vessels and used for suspension binding assays using ~0.25 nM ^{125}I-buserelin and the indicated concentration of unlabeled buserelin. Pooled K_d values were 2.4 ± 0.3 nM (n = 3) for buserelin binding to the hGnRH-R. The values shown are the mean ± S.E. normalized as a percentage of the binding seen with no competitor at an m.o.i. of 100. *Center panel,* WT dynamin cells cultured in 24 wells in the presence of tetracycline were infected with Ad hGnRH-R at m.o.i. values of 100 (○), 30 (●), 10 (△), and 3 (▲) and then cultured for a further 24 h. 2 μCi of [^{3}H]inositol was added to the medium for the final 16 h of culture after which the cells were washed and stimulated for 30 min with the indicated concentration of GnRH in the presence of 10 mM LiCl. Data shown are the mean ± S.E. of three experiments, each having duplicate determinations. The data was normalized by expressing ^{3}H eluted in the IP fraction as a percentage of that of free [^{3}H]inositol and IP fractions (*e.g.* fraction 3 as a % of fraction 1 + fraction 3). This provides an internal control for cell number and labeling efficiency and thereby facilitates pooling of data from repeated experiments. *Lower panel,* WT dynamin cells were infected with Ad hGnRH-R at m.o.i. values of 0 (○), 3 (●), 10 (△), 30 (▲), and 100 (▽) and then cultured for 18 h before being loaded with fura-2 and used for Ca^{2+} imaging. During imaging the cells were stimulated with 10^{-7} M GnRH as indicated. Each trace shows mean ± S.E. from three separate imaging experiments (n = 3), each using 10–50 cells.

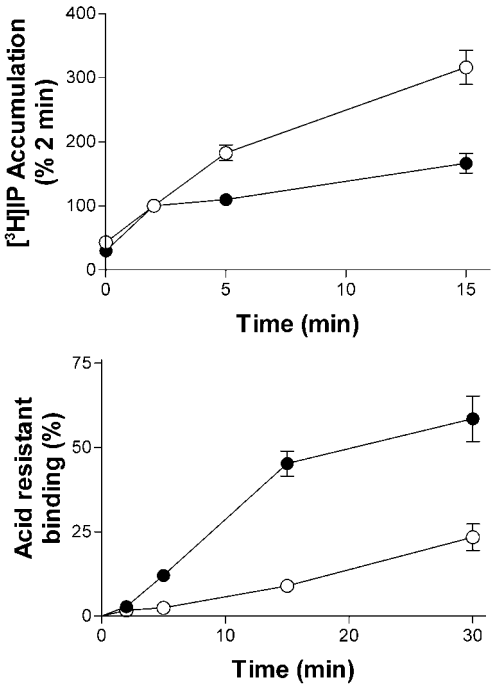

FIG. 2. **Time course of [^{3}H]IP accumulation and internalization in WT dynamin cells infected with Ad hGnRH-R or Ad XGnRH-R.** *Upper panel*: WT dynamin cells cultured in 24 wells in the presence of tetracycline were infected with Ad hGnRH-R (*open symbols*) or Ad XGnRH-R (*closed symbols*) at m.o.i. values of 50–100 and then cultured for a further 24 h. 2 μCi of [^{3}H]inositol was added to the medium for the final 16 h of culture after which the cells were washed and stimulated for the indicated time with the 10^{-7} M GnRH (hGnRH-R) or cGnRH-II (XGnRH-R), in the presence of 10 mM LiCl. The data are mean ± S.E. (n = 4–7) of pooled data normalized as a percentage of the [^{3}H]IP accumulation at 2 min. *Lower panel,* WT dynamin cells cultured in 24 wells in the presence of tetracycline were infected with Ad hGnRH-R (*open symbols*) or Ad XGnRH-R (*closed symbols*) at m.o.i. values of 50–100 and then cultured for a further 24 h. Cells were washed and incubated for the indicated period of time in PSS containing ~0.25 nM ^{125}I-buserelin (hGnRH-R) or 0.125 nM ^{125}I-cGnRH-II (XGnRH-R) with 0 or 10^{-6} M buserelin or cGnRH-II (nonspecific binding). The binding was terminated by transfer to ice-cold PSS and washing before solubilization of the cells in 0.2 M NaOH and 1% SDS. In a parallel experiment, cells were treated exactly as described above except that, after binding, the cells were incubated in acid medium to remove radioligand binding from cell surface receptors. Data shown are mean ± S.E. (n = 3) of specific binding (nonspecific binding subtracted) pooled from repeated experiments, each having triplicate observations that were expressed as a percentage of total specific binding (acid-labile plus acid-resistant) at 30 min.

or ^{125}I-cGnRH-II in cells infected with Ad hGnRH-R or Ad XGnRH-R, respectively (both at an m.o.i. of 100). The rank order of potency for competition at the hGnRH-R was buserelin>GnRH>cGnRH-II, whereas that at the XGnRH-R was cGnRH-II≫buserelin≫GnRH (not shown). Identical rank orders of potency were seen when HeLa cells infected with Ad hGnRH-R or Ad XGnRH-R were used to construct dose-response curves for [^{3}H]IP accumulation (not shown).

We next investigated desensitization of these receptors. To do so WT dynamin cells were infected with either Ad hGnRH-R or Ad XGnRH-R (at m.o.i. values of 100) and then used in time-course experiments in which [^{3}H]IP accumulation was measured. As shown (Fig. 2, *upper panel*) both receptors mediated comparable initial rates of [^{3}H]IP accumulation, but this initial rate was not maintained beyond 2 min for XGnRH-Rs stimulated by cGnRH-II. As an index of desensitization, the rate of accumulation between 2–5 min was only 9% ± 3% of the initial rate (0–2 min) for the XGnRH-R but was as high as 73% ± 15% for the hGnRH-R.

To monitor receptor internalization, WT dynamin cells were infected with either Ad hGnRH-R or Ad XGnRH-R (m.o.i. 100) and used in time-course experiments in which both total specific binding and acid resistant-specific binding were measured. Both ligands associated rapidly with their receptors with half-maximal binding at 5–10 min (not shown). However, the acid-resistant fraction of specific binding was greater in cells expressing XGnRH-Rs than hGnRH-Rs at all time points (Fig. 2, *lower panel*). To test whether these receptors stimulate fluid phase endocytosis, uptake of horseradish peroxidase was quantified in cells stimulated for 60 min with 0 or 10^{-7} M GnRH. Enzyme uptake was temperature-dependent (increased 2–3-fold by increasing from 4 °C to 37 °C) but was not measurably influenced by GnRH in Ad hGnRH-R-infected cells (93 ± 8% of control, $n = 4$) or by cGnRH-II in Ad cGnRH-II-infected cells (109 ± 9% of control, $n = 4$).

We next determined the effectiveness of the K44A dynamin blockade of internalization using the endogenous EGF receptors as controls. Pretreatment with EGF caused a time-dependent loss of cell surface EGF receptors (as judged by subsequent whole cell binding assays at 4 °C). In K44A dynamin cells cultured with tetracycline, surface binding was reduced to 26.8% ± 10.4%, 16.2% ± 1.3%, 9.1% ± 2.9%, and 8.1% ± 1.1% of control after 5, 15, 30, and 45 min, respectively (not shown). In contrast, no significant loss was seen in cells cultured in the absence of tetracycline (conditions permissive for expression of the dominant negative transgene). The specificity of this effect was established by control experiments in which EGF pretreatment caused a clear loss of cell surface receptors in WT dynamin HeLa cells, irrespective of the absence or presence of tetracycline in the culture medium.

The dynamin dependence of GnRH-R internalization was next investigated by infecting K44A dynamin cells with Ad hGnRH-R or Ad XGnRH-R, culturing these cells in the presence or absence of tetracycline, and then quantifying receptor internalization at 37 °C as above. As shown (Fig. 3, *upper panel*), ^{125}I-buserelin internalization (hGnRH-R-mediated) proceeded relatively slowly in control cells (cultured with tetracycline) to a maximum at 120 min with a half-time of 30–60 min. Omission of tetracycline from the culture medium (permissive for K44A dynamin expression) did not measurably influence this time-course. ^{125}I-cGnRH-II internalization (via XGnRH-R) was relatively rapid in control cells cultured with tetracycline (no measurable increase after 30 min and a half-time of ~10 min) and was clearly reduced in cells cultured without tetracycline (Fig. 3, *lower panel*). In control experiments (not shown), tetracycline had no direct effect on radioligand binding to either of these GnRH-R, and omission of tetracycline from the culture medium did not measurably influence internalization of either receptor in cells expressing wild-type dynamin (under tet-off control).

To determine whether differences in receptor number underlie the observed differences in dynamin dependence, internalization was assessed in cells infected with Ad hGnRH-R or Ad XGnRH-R at varied m.o.i. (from 3–100) and cultured with and without tetracycline. Fig. 4 shows the relationship between total specific binding (an index of receptor number) and acid-resistant binding (an index of receptor internalization) using data accumulated over four separate experiments. As expected, XGnRH-R internalization was greater than that for the hGnRH-R (72 ± 4% ($n = 28$) and 23 ± 2% ($n = 25$), respectively, data pooled irrespective of m.o.i.), and omission of tetracycline reduced the rate of XGnRH-R internalization (to 17 ± 2%, $n = 30$, $p < 0.05$) without altering that of the hGnRH-R ($p > 0.1$). Linear regression revealed that the rate of internalization of the XGnRH-R reduced as receptor number increased (gradient

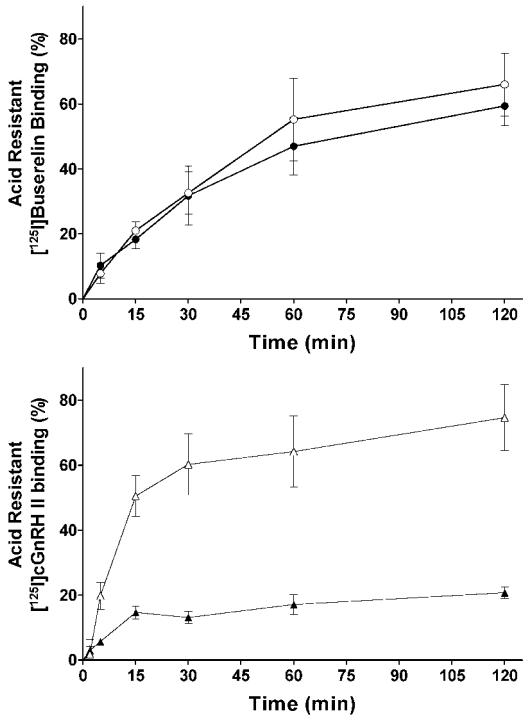

FIG. 3. **Dynamin dependence of XGnRH-R and hGnRH-R internalization in K44A dynamin cells.** K44A dynamin cells cultured in 24 wells in the presence of tetracycline were infected with Ad hGnRH-R (*upper panel*) or Ad XGnRH-R (*lower panel*) at m.o.i. values of 50–100. HeLa (K44A) cells were then cultured with (*open symbols*) or without (*closed symbols*) tetracycline for 8 h to allow infection before the medium was changed to fresh containing no Ad with or without tetracycline to allow expression of the mutant dynamin and then cultured for a further 16 h. Cells were washed and incubated for the indicated period of time in PSS containing ~0.25 nM ^{125}I-buserelin (hGnRH-R) or 0.125 nM ^{125}I-cGnRH-II (XGnRH-R) with 0 or 10^{-6} M buserelin or cGnRH-II (nonspecific binding). Transfer to ice-cold PSS and washing in PSS (total binding) or acid (internal binding) terminated the binding. The data shown are mean ± S.E. ($n = 3$) of acid-resistant binding expressed as a percentage of total specific binding pooled from repeated experiments, each having duplicate observations.

different from 0 at $p < 0.05$), but most importantly, the dynamin dependence of XGnRH-R internalization was maintained at a range of receptor densities encompassing those at which the hGnRH-R showed no dynamin dependence (Fig. 4).

We next addressed the dependence of GnRH-R internalization on CCVs using hypertonic sucrose (Fig. 5), which prevents the formation of clathrin lattices on coated pits and thereby prevents CCV-mediated receptor internalization (31). Using 15-min incubations, 72% ± 8% of ^{125}I-EGF binding was acid-resistant under control conditions, but this was reduced to 30% ± 5% ($p < 0.05$) by tetracycline omission (K44A expression). Similarly 51% ± 7% of ^{125}I-cGnRH-II binding to Ad XGnRH-R-infected cells was acid-resistant under control conditions, but this was reduced to 19% ± 4% ($p < 0.05$) by allowing K44A expression. In contrast, only 22% ± 4% of ^{125}I-buserelin binding was acid-resistant in control Ad hGnRH-R-infected cells, and this was not measurably altered by allowing K44A expression. Hypertonic sucrose (0.4 M) reduced the acid-resistant binding of all three ligands, to less than 5% without altering total specific binding (not shown), irrespective of the presence or absence of tetracycline (not shown). These data suggest that CCVs mediate dynamin-dependent and -independent internalization of GnRH-R.

Since GnRH is known to activate MAP kinase signaling in pituitary cells and GPCR-mediated MAP kinase activation can be dynamin-dependent, we next explored the possible effects on ERK 2 phosphorylation in Ad GnRH-R-infected K44A dynamin

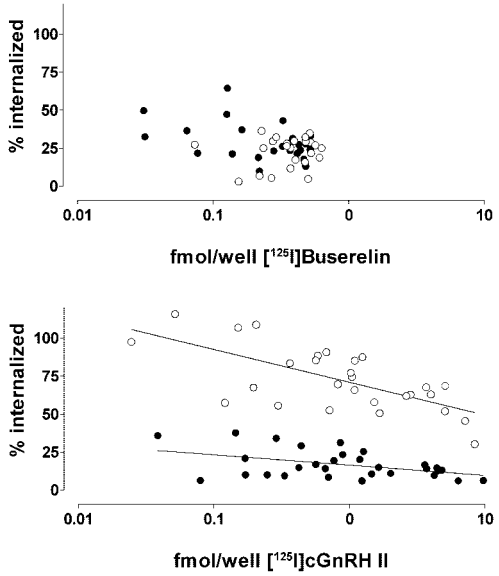

FIG. 4. **Density and dynamin dependence of XGnRH-R and hGnRH-R internalization in K44A dynamin cells.** K44A dynamin cells cultured in 24 wells in the presence of tetracycline were infected with Ad hGnRH-R (*upper panel*) or Ad XGnRH-R (*lower panel*) at m.o.i. values of 3–100. HeLa (K44A) cells were then cultured with (*open symbols*) or without (*closed symbols*) tetracycline for 8 h to allow infection before the medium was changed to fresh containing no Ad with or without tetracycline to allow expression of the mutant dynamin and then cultured for a further 16 h. They were then used in whole cells receptor internalization assays as above (Fig. 3 legend). The figures show total specific binding (an index of receptor number, *x* axis) plotted against the proportion of binding that was acid-resistant (% internalized, *y* axis). The data are pooled from multiple experiments, with each dot representing a single determination of each parameter from a single internally controlled experiment with triplicate replicates. Linear regression revealed a significant negative correlation of log transformed binding data and internalization data for the XGnRH-R with and without tetracycline (slope different from 0 at $p < 0.05$ in both cases) but not for the hGnRH-R ($p > 0.1$ in both cases).

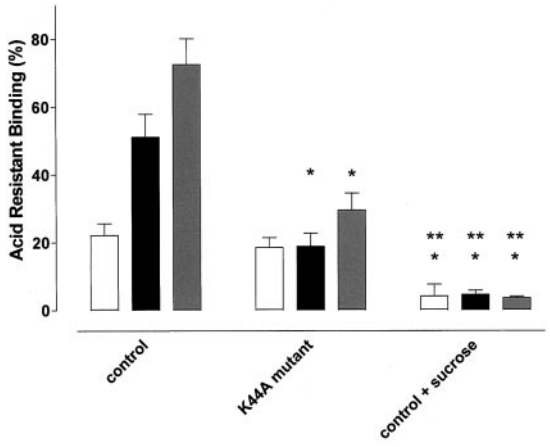

FIG. 5. **Effect of sucrose on the internalization of XGnRH-R and hGnRH-R in K44A dynamin cells.** K44A dynamin cells cultured in 24 wells in the presence of tetracycline were infected with Ad hGnRH-R (*open bars*) or Ad XGnRH-R (*black bars*) at m.o.i. values of 50–100 or left uninfected for EGF-R (*gray*). K44A dynamin cells were then cultured with (control cells) or without (K44A mutant) tetracycline for 8 h to allow infection before the medium was changed to fresh containing no Ad with or without tetracycline and then cultured for a further 16 h. Cells were washed and pretreated with PSS with or without 0.4 M sucrose for 30 min before radioligand binding and internalization for 15 min. Transfer to ice-cold PSS and washing in PSS (total binding) or acid (internal binding) terminated the binding. The data shown are mean ± S.E. (n = 3–12) of acid-resistant binding expressed as a percentage of total specific binding pooled from repeated experiments, each having triplicate observations (* $p < 0.05$ compared with control, ** $p < 0.05$ compared with K44A dynamin inhibited response).

cells cultured with and without tetracycline. As shown (Fig. 6), GnRH and cGnRH-II both caused pronounced activation of ERK 2 phosphorylation in control cells (expressing hGnRH-Rs and XGnRH-Rs, respectively) with comparable time-courses (maxima achieved at ~10 min. with subsequent reduction to near basal levels at 60 min). Stimulation of ERK 2 phosphorylation was also seen in cells cultured without tetracycline, but for both receptors the response was significantly reduced (area under the curve 75.9% ± 5.8 and 73.6% ± 7.4% ($p < 0.05$) of control for XGnRH-Rs and hGnRH-Rs, respectively). Thus, although the internalization of the hGnRH-R is apparently not dependent upon dynamin in HeLa cells, its effect on ERK 2 phosphorylation clearly is.

To explore the relationship between receptor internalization and signaling, we blocked internalization with sucrose and tested for possible inhibition of GnRH-R-mediated ERK 2 activation. However, these experiments were complicated by the fact that sucrose itself activated ERK 2 (not shown) and we therefore sought an alternative means of blocking internalization. As shown (Fig. 7), the lectin, concanavalin A, had no measurable effect on XGnRH-R-mediated ERK 2 phosphorylation in control cells (not expressing K44A dynamin), despite the fact that it reduced ^{125}I-cGnRH-II internalization by ~70% (from 68 ± 4% (n = 4) to 17 ± 1% (n = 5), $p < 0.05$). Similar results (inhibition of internalization but not of signaling) were obtained with Ad hGnRH-R infected cells (not shown).

To further explore the mechanism of ERK 2 activation in these cells, effects of PKC activation and inhibition were determined. As shown (Fig. 8), the PKC activator PMA (like GnRH and cGnRH-II) increased ERK 2 phosphorylation in control cells (not expressing K44A dynamin). PMA had no significant effect in the presence of the PKC inhibitor bisindolylmaleimide 1 (demonstrating the efficiency of the blocker), and the responses to activation of hGnRH-Rs and XGnRH-Rs were both inhibited by the inhibitor (to 50% ± 8 and 40% ± 8% ($p < 0.05$) of maximum, respectively), demonstrating a mediatory role for PKC. Expression of K44A dynamin inhibited hGnRH-R- and XGnRH-R-mediated ERK 2 phosphorylation as expected (to 26% ± 10 and 45% ± 7% ($p < 0.05$) of maximum stimulation, respectively) and also inhibited PMA-stimulated ERK 2 phosphorylation (to 59% ± 3%, $p < 0.05$), demonstrating that such inhibition is not GnRH-R-specific. When K44A dynamin was expressed, the dynamin-independent component of PMA-stimulated ERK 2 activation was blocked by bisindolylmaleimide 1 (again, demonstrating dependence on PKC activation), but we were unable to detect any such inhibition for responses to GnRH or cGnRH-II, implying that the dynamin-independent signaling of these receptors to ERK 2 is not PKC-mediated.

In a final series of experiments the effects of GnRH, cGnRH-II, and EGF were assessed in the presence and absence of the EGF receptor tyrosine kinase inhibitor AG1478. As shown (Fig. 9) EGF, GnRH, and cGnRH-II caused comparable phosphorylation of ERK 2 and were all sensitive to inhibition by expression of K44A dynamin (not shown). In contrast, 1000 nM AG1478 prevented EGF-stimulated ERK 2 phosphorylation without measurably altering the ERK 2 phosphorylation mediated by activation of either GnRH-R.

DISCUSSION

The GnRH receptor family has undergone a period of rapidly accelerated molecular evolution in which the C-terminal tails, present on all known non-mammalian GnRH-R, have been lost with the advent of mammalian type I GnRH-R. This provides a unique opportunity to study the functional relevance of these features with normal (non-mutated) receptors. Such studies

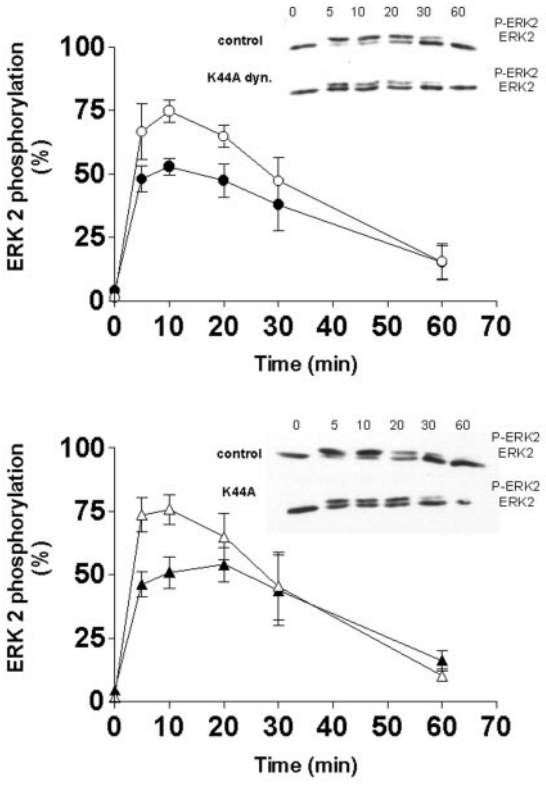

FIG. 6. **Effect of dynamin on the time course of ERK 2 activation in K44A dynamin cells infected with Ad hGnRH-R or Ad XGnRH-R.** Phosphorylation of ERK 2 was assessed by Western blotting to detect and distinguish unphosphorylated and phosphorylated ERK 2 by the retarded mobility of the latter in SDS-polyacrylamide gel electrophoresis (*inset*). HeLa (K44A) cells were infected with Ad hGnRH-R (*upper panel*) or Ad XGnRH-R (*lower panel*) at m.o.i. values of 50–100 before being cultured with (*open symbols*) or without (*closed symbols*) tetracycline. Cells were then stimulated with 10^{-7} M GnRH (hGnRH-R) or cGnRH-II (XGnRH-R) for the indicated period of time. The figure shows mean ± S.E. ($n = 4$) calculated after expressing the phosphorylated ERK 2 as a percentage of total ERK 2 (both determined by densitometry).

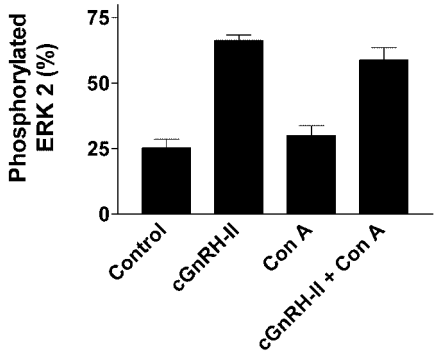

FIG. 7. **Influence of concanavalin A on XGnRH-R-mediated ERK 2 activation.** ERK 2 phosphorylation was determined and quantified as described above in K44A HeLa cells infected with Ad XGnRH-R at an m.o.i. of 100. The cells were pretreated with 0 or 0.25 mg/ml concanavalin A (*Con A*) for 30 min prior to stimulation for 15 min with 0 or 10^{-7} M cGnRH-II as indicated. The figure shows mean ± S.E. ($n = 3$) calculated after expressing the phosphorylated ERK 2 as a percentage of total ERK 2 (both determined by densitometry). ERK 2 phosphorylation was not measurably influenced ($p > 0.1$) by concanavalin A in the presence or absence of cGnRH-II. Similar data were obtained in cells infected with Ad hGnRH-R and stimulated with 0 or 10^{-7} M GnRH (not shown).

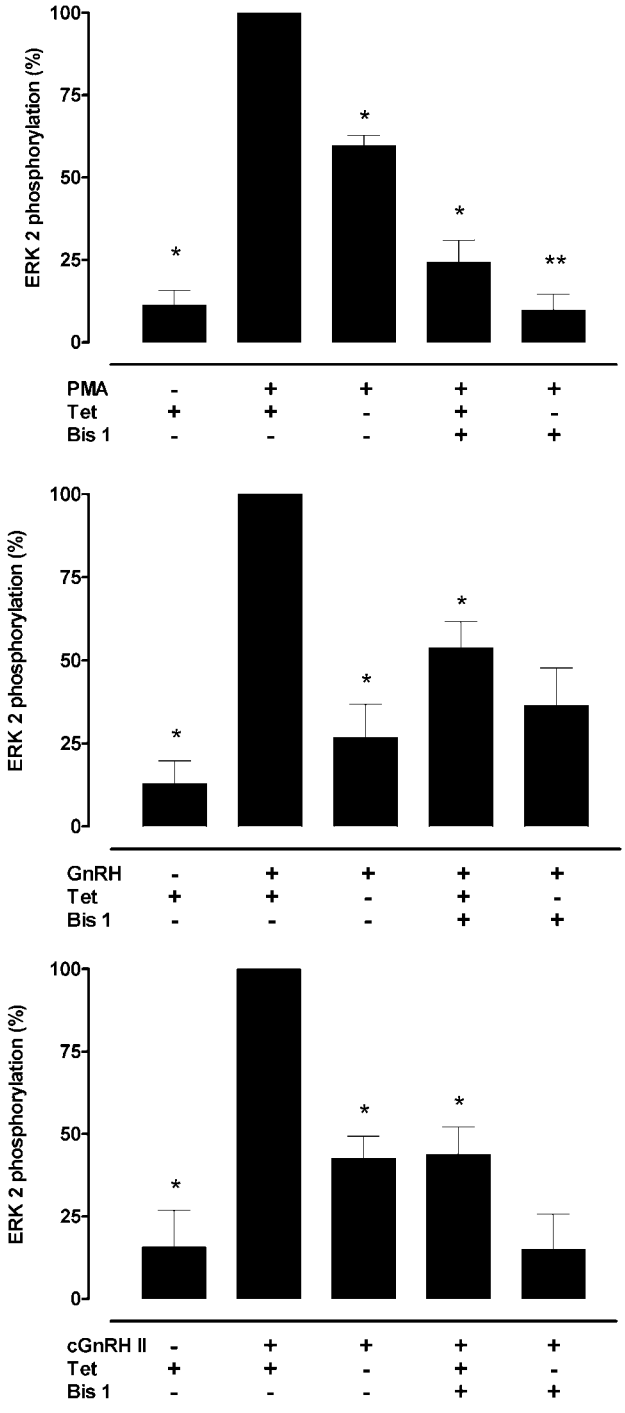

FIG. 8. **PKC-dependence of GnRH-R-mediated activation of ERK 2 in K44A dynamin cells infected with Ad hGnRH-R or Ad XGnRH-R.** K44A dynamin cells were infected with Ad hGnRH-R or Ad XGnRH-R at m.o.i. values of 50–100 before being cultured with or without tetracycline. Cells were then pretreated with PSS with or without 1 μM bisindolylmaleimide 1 for 30 min before being stimulated with PSS containing 30 nM PMA (*top panel*), 100 nM GnRH (hGnRH-R, *middle panel*) or cGnRH-II (XGnRH-R, *bottom panel*) for 10 min before extraction. Data is expressed as a percentage of maximal stimulation determined by the proportion of ERK 2 that is in the phosphorylated form from pooled observations ($n = 3$), as determined by densitometry measurement. The PMA control is pooled from both hGnRH-R- and XGnRH-R-infected cells ($n = 6$) (* $p < 0.05$ compared with maximal stimulation (no inhibitor), ** $p < 0.05$ compared with both maximal stimulation and dynamin blocked stimulation).

have revealed that mammalian GnRH-R do not rapidly desensitize whereas the two non-mammalian GnRH-R investigated to date (catfish (23) and *Xenopus* GnRH-R (26)) do show rapid homologous desensitization. Similarly, mammalian GnRH-Rs undergo agonist-induced internalization, but at much lower rates than non-mammalian GnRH-R (24, 26, 27). The resist-

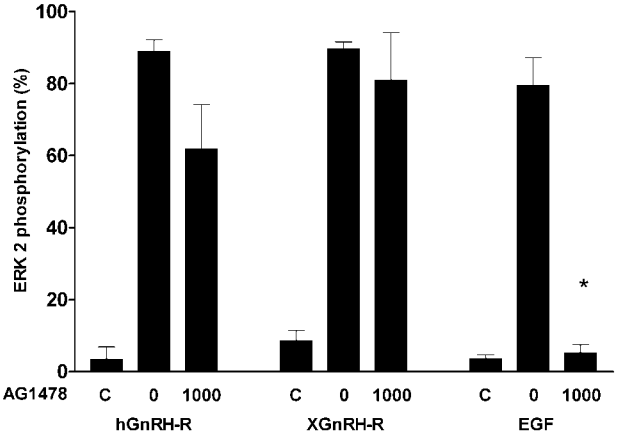

FIG. 9. **Tyrosine kinase dependence of GnRH-R-mediated activation of ERK 2 in K44A dynamin cells infected with Ad hGnRH-R or Ad XGnRH-R.** K44A dynamin cells were infected with Ad hGnRH-R or Ad XGnRH-R at m.o.i. values of 50–100 before being cultured with tetracycline. Cells were then pretreated with PSS with or without 1000 nM AG1478 for 30 min before being stimulated with PSS containing 10^{-7} M GnRH (hGnRH-R) or cGnRH-II (XGnRH-R) or 10^{-7} M EGF (EGF-R) for 10 min before extraction. Data is expressed as the percentage of ERK 2 that is in the phosphorylated form from pooled observations ($n = 3$) as determined by densitometry measurement. * $p < 0.05$ compared with maximal stimulation (no inhibitor).

ance of mammalian GnRH-R to desensitization and internalization has been attributed to the lack of required C-terminal tail phosphorylation sites since catfish GnRH-R do undergo agonist-induced phosphorylation and bind β-arrestin (causing it to translocate to the plasma membrane), whereas mammalian GnRH-R do not (32). Similarly, we have found that XGnRH-R mediate translocation of β-arrestin-GFP to plasma membranes, whereas hGnRH-R do not (not shown). Moreover, internalization of catfish GnRH-R is β-arrestin-dependent (24), whereas internalization of the human GnRH-R is not (25). Thus non-mammalian GnRH-Rs appear to follow the scheme outlined above for β-arrestin-mediated desensitization and internalization (and possible β-arrestin-mediated signaling), but this is not the case for the mammalian GnRH-Rs investigated to date (20, 22).

Here we have explored the dynamin dependence of desensitization, internalization, and signaling of human and *Xenopus* GnRH-Rs using recombinant adenovirus to express these receptors in HeLa cells expressing either wild-type or K44A (dominant negative) dynamin. GnRH-Rs expressed in this way had high affinity for receptor-specific ligands (low nM K_d for ^{125}I-buserelin binding to hGnRH-Rs and for ^{125}I-cGnRH-II binding to XGnRH-Rs) and mediate PLC activation, Ca^{2+} mobilization, and MAP kinase (ERK 2) activation. They also show different ligand specificity (buserelin>GnRH>cGnRH-II at hGnRH-Rs and cGnRH-II≫buserelin>GnRH at XGnRH-Rs) and different rates of internalization and desensitization (both being more rapid for the XGnRH-R). Receptor density, and receptor-mediated effects on [^{3}H]IP accumulation and $[Ca^{2+}]_i$ were all increased by increasing viral titer from m.o.i. values of 3–100, and Ca^{2+} imaging experiments revealed that ~90% of the cells were GnRH-responsive after infection at an m.o.i. of 10. Accordingly, the increases in hGnRH-R number caused by increasing m.o.i. from 10 to 100 (14,600–46,500 receptors/cell) reflects an increase in receptors/cell rather than in the proportion of cells expressing the receptor. These values compare well to the range of densities of endogenous mammalian GnRH-Rs in pituitary gonadotrophs (33). Thus, recombinant Ad provide an efficient means of expressing GnRH-Rs in HeLa cells at physiological density, and these receptors retain the pharmacological characteristics anticipated from earlier studies with endogenous GnRH-Rs and GnRH-Rs expressed heterologously in pituitary gonadotrope progenitor cells (26, 34, 35).

The dynamin dependence of EGF receptor internalization has previously been demonstrated in K44A dynamin cells (3) and was confirmed here as a positive control for blockade of dynamin-dependent endocytosis. Using this system, we have found, as expected, that internalization of the XGnRH-R is more rapid than that of the hGnRH-R in K44A dynamin cells cultured with tetracycline. Blockade of dynamin-dependent internalization by tetracycline omission did not reduce hGnRH-R internalization. This was unexpected because transient expression of K44A dynamin has been reported to inhibit internalization of the rat GnRH-R by 20% in COS 7 cells (32). Although a tendency for inhibition was observed at the 60- and 120-min time points (Fig. 3), and in some subsequent experiments (*e.g.* Fig. 4), this did not attain statistical significance (even when data were pooled from multiple series of experiments), and we were therefore unable to demonstrate any measurable dynamin dependence of hGnRH-R internalization in HeLa cells. In stark contrast, the internalization of XGnRH-Rs was dramatically reduced when dynamin-dependent internalization was prevented by tetracycline omission.

Internalization of several GPCRs is inhibited by expression of dominant negative mutants of dynamin (6, 15) and by prevention of CCV formation using hypertonic sucrose (31). Although these treatments are not entirely specific for CCVs (4, 11, 37, 38), electron microscopy has revealed that mammalian GnRH-R are internalized via coated vesicles (39) and co-internalization with transferrin (a marker for CCV-mediated internalization) implies that these are CCVs (25). We have recently found that the XGnRH-R also co-internalizes with transferrin (not shown). Accordingly, the sucrose-dependent internalization of both GnRH-R in HeLa cells is also most likely CCV-mediated.

We were concerned that the observed differences in GnRH-R internalization might reflect differences in receptor number and tested this by expressing receptors at varied density (varying viral titer between m.o.i. values of 3–100). This revealed a negative correlation between XGnRH-R binding and internalization rate (Fig. 3), supporting the notion that the stoichiometry of receptors and other proteins can indeed influence the observed internalization. However, the key distinctions between hGnRH-R and XGnRH-R internalization (faster internalization and dynamin sensitivity of the later) are maintained over a wide range of receptor density (Fig. 4). The retention of these distinctions with a range of XGnRH-Rs estimated to encompass that of the hGnRH-Rs clearly demonstrates dependence on receptor structure rather than receptor density.

The best-explored pathway for ERK 2 activation by tyrosine kinase receptors involves Shc/Grb2-mediated activation of a Ras guanine nucleotide exchange factor that activates the monomeric G-protein, Ras. This in turn activates Raf, which phosphorylates MEK causing it to phosphorylate ERK 2. Numerous GPCRs, including GnRH-Rs, have also been shown to activate ERK 2 by feeding into this pathway at multiple sites (41, 42). Up-stream activation can be achieved by GPCR-mediated transactivation of EGF receptor involving stimulated liberation of HB-EGF in the extracellular environment (19). For Gq-coupled GPCRs activation of PKC can lead to Raf activation, and Ca^{2+} elevation can activate a Ras G-protein regulatory factor. More recently, β-arrestin, recruited to β_2-adrenergic receptors, was shown to bind Src, which can regulate Shc/GRb2 (12, 36, 43), and the observation that dominant negative mutants of β-arrestin or dynamin-blocked ERK 2 activation implied that GPCR endocytosis was necessary for signaling to the MAP kinase. These observations are intriguing, not only

because they demonstrate signaling of the "desensitized" receptor, but also because Src phosphorylates and activates dynamin (44), providing a mechanism for activation of dynamin-dependent internalization by the GPCR. However, EGF receptor signaling and MEK signaling are also dynamin-dependent (3, 16), so the dynamin dependence of GPCR signaling can actually reflect the fact that GPCR activation impinges on the EGF receptor/ERK 2 signaling pathway (reviewed in Ref. 42).

From these observations, it is clear that understanding the relationship between GPCR cycling and ERK 2 activation will depend upon the loci at which the GPCR signaling pathway feeds into the MAP kinase cascade. Here, we have found that hGnRH-R and XGnRH-R both mediate ERK 2 phosphorylation (indicative of activation of the ERK 1/2 signaling cassette) in K44A cells cultured with tetracycline. To our knowledge, this is the first demonstration of MAP kinase activation by a non-mammalian GnRH-R, and it is therefore of interest that time-courses and amplitudes of the responses were indistinguishable. This clearly implies that rapid GPCR desensitization (seen for the XGnRH-R but not for the hGnRH-R) is not an important determinant of the kinetics of this response. It remains to be determined whether β-arrestin-mediated signaling (which may occur for the XGnRH-R but presumably not for the hGnRH-R) supports the response to XGnRH-R activation.

In addressing the mechanisms of ERK 2 activation we found that the tyrphostin AG 1478 abolished EGF-stimulated ERK 2 activation at a concentration that does not inhibit the response to activation of either of the GnRH-Rs used. ERK activation by the endogenous mouse GnRH-R of αT3–1 pituitary cells has also been shown to be resistant to inhibition by AG1478 and by dominant negative EGF receptors (40) despite the fact that earlier work had implied a role for EGF receptor transactivation in GnRH action (18). In the HeLa cells used here, the differential sensitivity to AG1478 clearly implies that EGF-R activation (17–19)) does not underlie GnRH-R-mediated ERK 2 activation. However, we have also found that activation of ERK 2 by both GnRH-Rs was partially inhibited by dynamin despite the fact that internalization of the hGnRH-R was not. This uncoupling clearly argues that dynamin-dependent internalization of the GPCR is not required for dynamin-dependent ERK 2 activation.

The interpretation above is reinforced by the fact that inhibition of XGnRH-R internalization with concanavalin A did not reduce XGnRH-R-mediated ERK 2 activation. Moreover, we found that PMA-stimulated ERK 2 activation is also dynamin-dependent and that the PKC inhibitor (bisindolylmaleimide 1) inhibited ERK 2 activation by both GnRH-Rs. Although multiple mechanisms are likely to be involved, the simplest interpretation of these data is that GnRH-R-mediated ERK 2 activation is largely PKC-mediated in these cells and that it is dynamin-dependent because signaling from PKC to ERK 2 is, at least in part, dynamin-dependent. Recent studies in pituitary cells indicate that GnRH activates ERK primarily by causing a PKC-mediated activation of Raf with only a minor contribution from Src and Ras to Raf activation (40). GnRH-stimulated ERK activation was also found to be partially dynamin-sensitive in this system, but this effect was confined to inhibition of the Src/Ras signaling step (*e.g.* up-stream of, or unrelated to, PKC activation). However, this does not hold true in HeLa cells where events downstream of PKC are also dynamin-dependent (*e.g.* PMA-stimulated ERK 2 activation is inhibited by K44A dynamin). Signaling by MEK, which lies down-stream of PKC in the GnRH-R signaling cascade, has been shown to be dynamin-dependent in COS 7 cells (16). In this system, the dynamin-dependent endocytosis of activated MEK was critical for MAPK activation, and this relationship may well explain the dynamin dependence of GnRH-R signaling shown here.

In the preceding sections, inhibition by K44A dynamin has been equated with "dynamin dependence", but this may be an over-simplification because internalization of different GPCRs has been found to be differentially sensitive to distinct mutants of dynamin (11). This raises the possibility that the K44A dynamin-independent hGnRH-R internalization described here might prove sensitive to other dynamin mutants, but even if this is the case, the difference between the hGnRH-R and the XGnRH-R (and EGF receptor) in terms of sensitivity to K44A dynamin 1 implies that they access functionally distinct internalization routes.

In summary, a functional comparison of human and *Xenopus* GnRH-Rs has revealed that the tail-less mammalian GnRH-R internalizes and desensitizes slowly as compared with a tailed non-mammalian GnRH-R, and that this distinction is a function of receptor type rather than receptor density. Expression of K44A dynamin did not measurably alter desensitization or hGnRH-R internalization but dramatically reduced internalization of the XGnRH-R and EGF receptor, whereas blockade of CCV formation with sucrose abolished internalization of all three receptors. The tyrphostin, AG 1478, blocked EGF-stimulated, but not GnRH-R-mediated, ERK 2 activation, demonstrating that EGF receptor transactivation is not mediating GnRH action in these cells. K44A dynamin expression also inhibited ERK 2 activation mediated by either GnRH-R, whereas inhibition of GnRH-R internalization with concanavalin A did not influence GnRH-R-mediated ERK 2 activation. PMA also caused a dynamin-dependent activation ERK 2, and the effects of PMA, GnRH, and cGnRH-II on ERK 2 phosphorylation were all blocked by a PKC inhibitor. Thus we have established that a) desensitizing and non-desensitizing GnRH-Rs are targeted for CCV-mediated internalization by functionally distinct mechanisms, b) GnRH-R signaling to ERK 2 is dynamin-dependent, and c) this does not reflect a dependence on dynamin-regulated GnRH-R internalization but may instead be attributable to the dynamin dependence of PKC-mediated MEK activation.

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