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Evolved Regulation of Gonadotropin-Releasing Hormone Receptor Cell Surface Expression

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Dominant negative effects of mutant gonadotropin-releasing hormone (GnRH) receptors (GnRHR; isolated from patients with idiopathic hypogonadotropic hypogonadism) on plasma membrane expression (PME) and function of the wt GnRHR were examined. In addition, we assessed the effect of mutants on wt GnRHR with receptor modifications that, by themselves, diminished PME. Among such mechanisms that restrict PME of GnRHR in primates are: (a) addition of the primate-specific K^{191} and (b) deletion of the carboxyl tail ("C-tail") found in pre-mammalian species (fish, birds) of GnRHR. We prepared rat (r) and human (h) GnRHR plasmids (88% homologous), each with or without the K^{191}; chimeras were then made with C-tail or each of four truncated fragments (selected to isolate consensus sites for palmitoylation or phosphorylation) of the 51-amino-acid Ser-rich piscine GnRHR C-tail and then expressed in COS-7 cells. The data suggest that the dominant negative effect of the mutants on the hGnRHR requires intrinsic low PME that co-evolved with the dominant-negative effect. The data further reveal that additional modifications must have occurred in primates that are important for both the diminution of the PME and the development of the dominant negative effect of the mutants.

Key Words: GnRH receptor; G-protein coupled receptor; receptor targeting; membrane expression; receptor evolution; heptahelical receptor.

Introduction

The gonadotropin-releasing hormone (GnRH) receptor (GnRHR) is a member of the G-protein-coupled receptor (GPCR) superfamily *(1–4)*. Resistance to GnRH by loss-of-function mutants of the human (h) GnRHR gene leads to distinct forms of autosomal recessive hypogonadotropic hypogonadism (HH) *(5)*. Expression of thirteen naturally occurring hGnRHR point mutations, distributed over the entire sequence of the GnRHR, shows that these mutants lose function because most (at least 11) become *misrouted* proteins, rescuable by genetic *(6)* or pharmacological means *(7–10)*, rather than having intrinsic defects in ligand binding and/or G-protein coupling.

The GnRHR was the first member of its superfamily shown to activate upon dimerization *(11,12)*, an event that may be a general characteristic of a number of GPCRs *(13, 14)*. Some GPCRs dimerize as they are synthesized, a potential requisite for targeting to the cell membrane, whereas others are monomeric in the membrane and dimerize upon receptor activation *(13–15)*. In principle, association of GPCRs in the intracellular compartment could lead either to intracellular retention of the complex (a dominant-negative effect, as appears to be the case for the V_2-vasopressin, the platelet-activating factor, and the CCR5 chemokine receptors) *(16–18)* or to cell surface expression (dominant-positive effect, as is the case of the metabotropic $GABA_BR1$ and $GABA_BR2$ receptors) *(19,20)*. Furthermore, splice variants of the GnRH and D_3-dopamine receptors impair cell surface expression of their corresponding wild-type (wt) counterparts, presumably due to association and retention in the endoplasmic reticulum *(21–23)*. More recently, we have found that eight naturally occurring human GnRHR mutants, whose function may be rescued by pharmacological chaperones, exhibit dominant-negative actions on hwt GnRHR function, an effect that presumably occurs through formation of intracellular heterocomplexes between both receptor species *(24)*.

It is empirically observed that plasma membrane expression (PME) of the GnRHR decreases concurrently with higher stages of evolution, as exemplified by the differences in PME levels among fish, rodents, and primates *(9)*. Comparison of the structure of GnRHR from multiple species, along with creation of interspecific chimeras *(6)*, suggest that nature has used at least two distinct approaches to effect decreased membrane expression of this protein. First, pre-mammalian GnRHRs contain an intracytoplasmic "tail' at the carboxyl (C) terminus ("C-tail") *(25)*. Evolutionary

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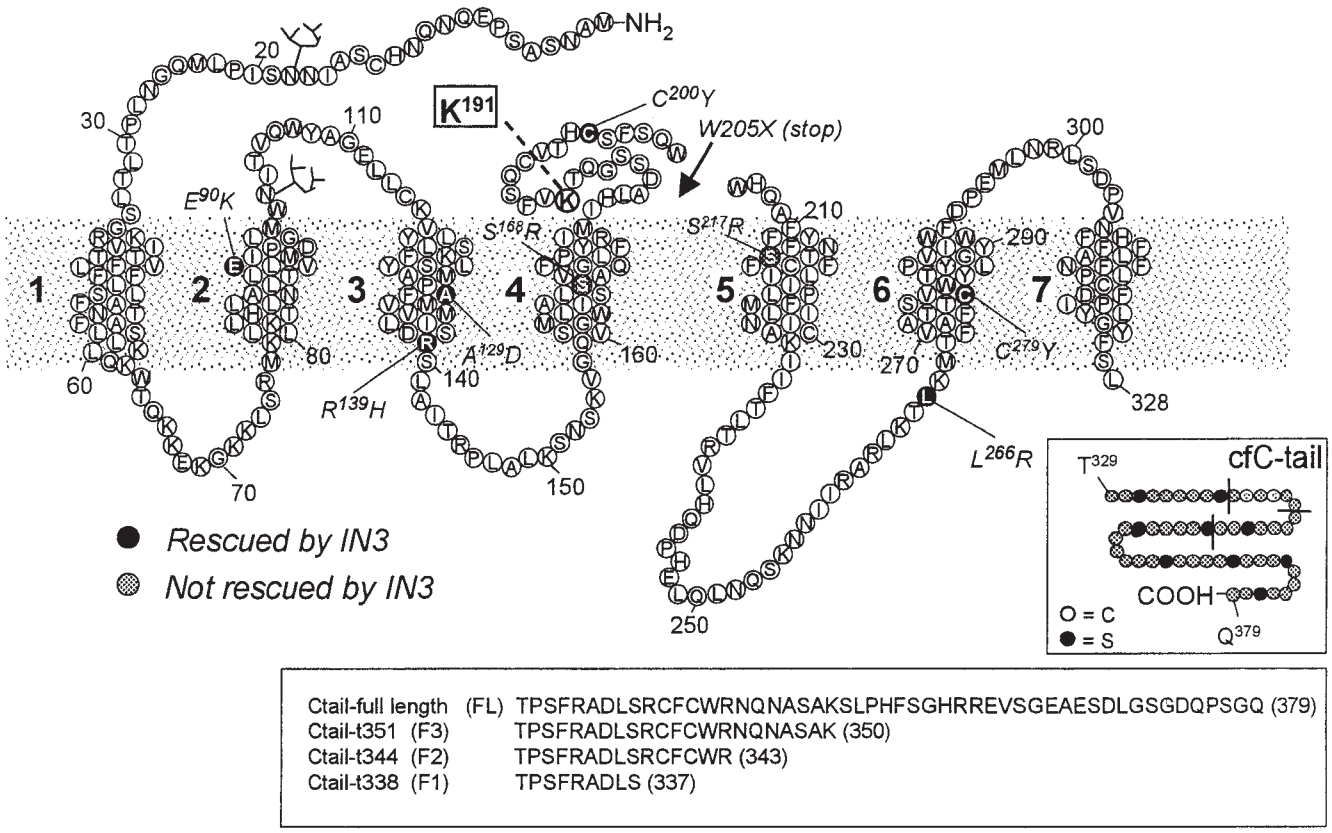

Fig. 1. Sequence of the human GnRH receptor and location of the inactivating mutations studied. Genetic modifications introduced in the hGnRHR [deletion of K^{191} in the second extracellular loop and addition of catfish carboxyl-terminal tail fragments (*insets*)] are also shown. IN3 is a peptidomimetic, cell-permeant GnRH antagonist and is an efficient pharmacological chaperone for misfolded hGnRHR mutants.

truncation of this sequence [51 amino acids in the catfish (cf), for example] is associated with decreased plasma membrane expression *(9,26)*, whereas production of a chimeric rat (r) GnRHR containing the C-tail of the cfGnRHR, leads to elevated expression *(26)*. This serine-rich (9 Ser of 51 amino acids) C-tail also contains a single consensus site for palmitoylation (Cys-X-Cys) *(27)*, as well as potential sites for ligand-induced phosphorylation *(28)*, which in many GPCRs are also associated with plasma membrane anchoring and receptor desensitization, respectively *(29)*. Second, primate GnRHR contains a specific K^{191} in its second extracellular loop, which is not found in pre-mammalians and that is associated with a further decrease in plasma membrane expression *(28)*. Deletion of this amino acid from the human GnRHR sequence results in enhanced membrane expression *(9,30,31)*, whereas its replacement by E or G (the corresponding residues present in GnRHRs from other mammals such as horse, pig, sheep and the silver-gray bush-tailed opossum) did not modify receptor expression or function *(30)*.

The present studies were undertaken in order to determine if (a) specific regions of the catfish C-tail with assignable functions (palmitoylation, phosphorylation) are responsible for the increased PME of receptors bearing this structural feature, (b) the dominant negative action of the HH mutants on wt receptor expression was intimately associated with the level of receptor function, and (c) the primate-specific K^{191} was sufficient to mediate the dominant-negative action of the mutants.

Results

Map of the wt hGnRHR Showing the Mutants and Chimeras Studied

Figure 1 is a map of the hGnRHR showing the localization of the primate specific K^{191}, the sequence of the C-tail fraction chimeras examined in the present study, and the site of the naturally occurring mutants and a truncation mutant (*W205X*) of the hGnRHR. In the present studies only the wt human and rat GnRHRs (not the point mutants shown) were genetically modified by addition or removal of K^{191} or C-tail (or its fragments). Individual mutants ($E^{90}K$, $A^{129}D$, $R^{139}H$, $S^{168}R$, $C^{200}Y$, $S^{217}R$, $L^{266}R$, $C^{279}Y$) were identified in patients with hypogonadotropic hypogonadism *(5,6,32–35)* and were coexpressed as described below without further genetic modification. The mutants do not show binding to ^{125}I-labeled GnRH agonist at the cell surface or coupling to the $G_{q/11}$ system when expressed indi-

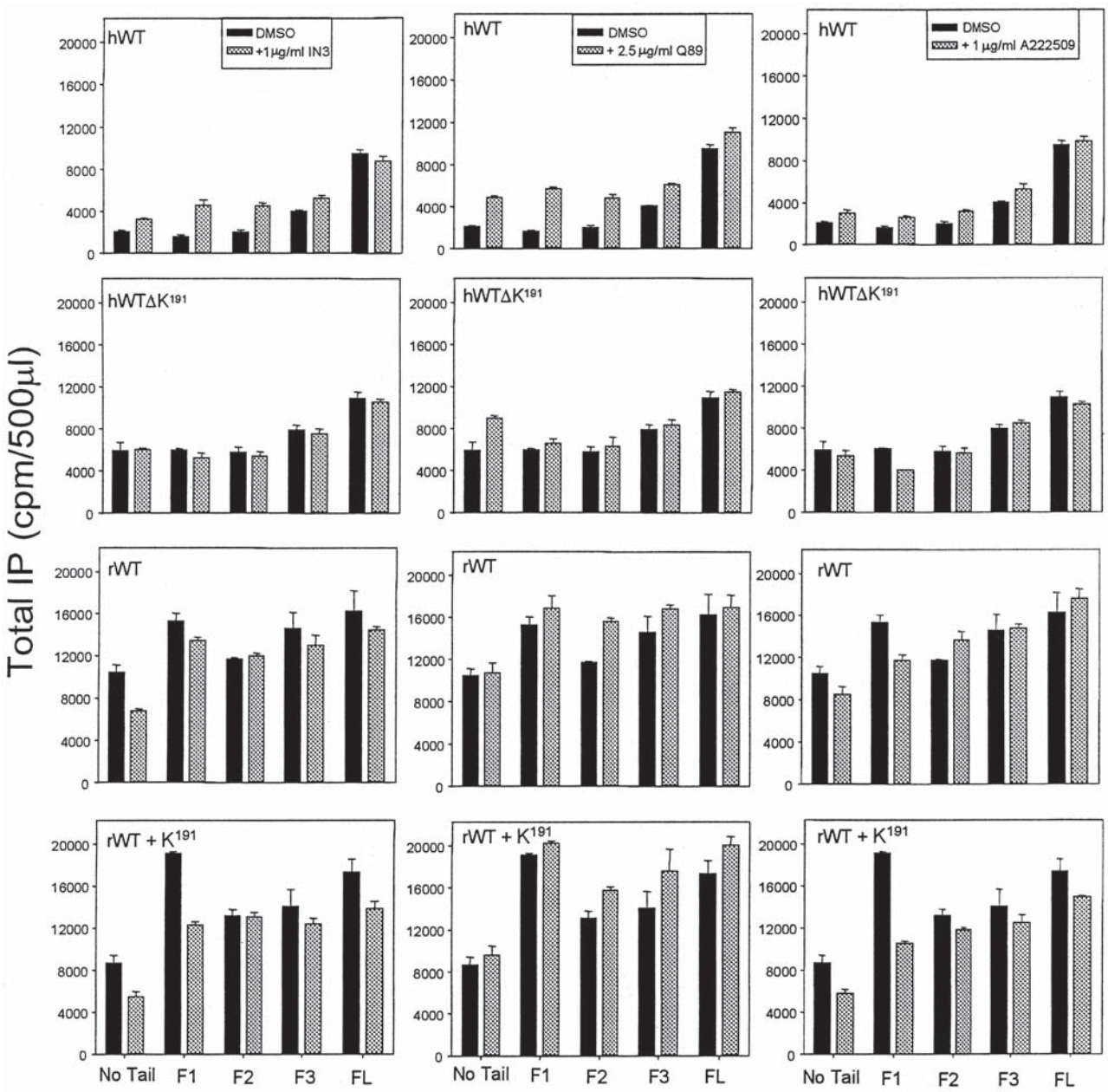

Fig. 2. Effect of the GnRH antagonists IN3, Q89, and A222509 on functional expression of GnRHR plasma membrane expression of various plasmids. COS-7 cells were transfected with the indicated plasmid as described in Materials and Methods and then incubated in the absence (■) or presence (▨) of the indicated compound. All culture wells were then incubated with 10^{-7} *M* buserelin and IP production measured as described in Materials and Methods. A similarly treated set of cells was incubated in medium without buserelin and showed only basal level IP production (not shown).

vidually in COS-7 cells and exposed to the GnRH agonist buserelin *(7,8)*. As noted, particular mutants can be rescued with the peptidomimetic agent, IN3, and are correctly routed to the plasma membrane where they couple to the effector system *(7,8,10)*.

Function of Genetically Modified GnRHR Constructs

Owing to the low amounts of cDNA transfected, which precluded a reliable measurement of the extent of GnRH binding, we used buserelin-stimulated inositol phosphate (IP) production as an indirect measure of the plasma membrane expression and effector coupling of GnRHRs; IP has been shown to reliably reflect membrane expression in previous studies *(7,8,24)*. We found that both hGnRHR and rGnRHR, each with and without the primate-specific K^{191} or the piscine-specific carboxyl tail, express at different levels when transfected into COS-7 cells (Fig. 2). Addition of the largest fragments (F3 and FL) of the cfC-tail to wt hGnRHR and hGnRHRΔK^{191} progressively increased total IP production, whereas the function of both wt rGnRHR

and rGnRHR+K^{191} increased by addition of all C-tail fragments. Interestingly, and in contrast to previous observations in GGH3 cells *(26)*, the function of the wt rGnRHR expressed in COS-7 cells was maximal when the shortest C-tail fragment (F1) was added. When the pharmacological chaperone IN3 was used to optimize the plasma membrane expression of the receptor by stabilizing the receptor structure that is not selected for degradation by the cell's quality control system *(8–10,36)*, the functional level of the hwt receptor and the hwt/C-tail-F1 and -F2 chimeric receptors markedly increased (Fig. 2). In contrast, IN3 had no effect on the hWTΔK^{191} receptor or any of the hWTΔK^{191}/C-tail fraction chimeras, whose function was already comparatively enhanced by the absence of K^{191} and the addition of the entire C-tail. While exposure to this pharmacoperone had no effect on the functional expression of rwt/C-tail fraction (F1 to F3 or FL) chimeras or the rwt receptor to which K^{191} and C-tail fractions F2, F3, and FL were added, the PME of the rwt receptor and rwt+K^{191} and rwt+K^{191}/C-tail-F1 modified receptors was attenuated. Similar results were found when the cells were exposed to the erythromycin-derived macrolide, A222509, but not the quinolone structure, Q89, which appeared to be a more effective chaperone in some cases. Although not totally surprising, because the molecule is very different in the chemical structure and, presumably, interacts differently with the GnRHR chimeras and has differences in solubility and permeability *(36)*, the precise reason for this disparity is not immediately obvious.

Co-expression of Human or Rat wt and Genetically Modified GnRHRs with hGnRHR Mutants

wt hGnRHR

Compared to the genetically modified forms of the hGnRHR and the rGnRHR as well as to the rwt receptor, wt hGnRHR is the most modestly expressed as it both contains the K^{191} and lacks the piscine-specific C-tail (Figs. 1 and 2). When expressed alone in COS-7 cells, the nine mutant receptors studied, do not measurably bind GnRH agonists or couple to the $G_{q/11}$ system *(7,8)*. These mutants show a dominant-negative action when co-expressed with the wt hGnRHR, an effect that is markedly ameliorated by addition of the complete C-tail (FL) to the wt receptor (Fig. 3e and j). For some mutants ($A^{129}D$, $R^{139}H$ and $L^{266}R$), increasing tail lengths are associated with progressively increased functional expression of the wt receptor and loss of the dominant-negative effect. Of note, the relative potency of each of the mutants to subserve a negative effect on wt receptor function is largely maintained as the C-tail is lengthened until the complete C-tail is added, a condition at which the dominant-negative effect is markedly attenuated ($E^{90}K$, $A^{129}D$, $S^{168}R$, and $C^{279}Y$) or virtually disappears ($R^{139}H$, $C^{200}Y$, $S^{217}R$ and $L^{266}R$) (Figs. 3a–e). Two mutants that cannot be rescued by the pharmacological chaperone IN3, $S^{168}R$ and $S^{217}R$ *(7,8)*, appear to be among the most potent in exerting the dominant-negative effect on the hwt receptor.

hGnRHΔK^{191}

Removal of the primate-specific K^{191} from the wt hGnRHR, results in enhanced plasma membrane expression (compared to wt hGnRHR, Fig. 3) and substantial loss of the dominant negative effect of the mutants (Fig. 4). Addition of the C-tail fragments or the full C-tail does not have a consistent effect on the functional level of PME of the wt hGnRHRΔK^{191}, but again, the relative potency of each mutant is preserved. Interestingly, co-transfection of hwtΔK^{191} with the $R^{139}H$ or $C^{200}Y$, consistently increased the $G_{q/11}$ coupling efficiency of the hwtΔK^{191} function above control levels (Fig. 4a).

wt rGnRHR

The wt rGnRHR (normally) lacks a K^{191} and is expressed at a fivefold higher level compared to wt hGnRHR; this level is further increased by the C-tail fragments, particularly the F1, F3, and FL fragments (Fig. 2). The functional level of this receptor is only slightly altered when co-expressed at any tail length with the hGnRHR mutants (Fig. 5). Without C-tail substitution, a clear dominant positive action of particular mutants ($R^{139}H$, $S^{168}R$, $C^{200}Y$, $S^{217}R$, and $W^{205}X$) is observed.

rGnRHR plus K^{191}

Addition of the K^{191} to the rat wt receptor sequence produces a functional level that is only slightly above the human sequence lacking this amino acid and below that for the rwt (Fig. 2). Addition of C-tail fragments, particularly the F1 and the complete C-tail, elevates IP production by the modified receptor (Fig. 2). Several human receptor mutants show modest dominant positive effects in the absence of C-tail or when the F1 fragment is added to the wt rGnRHR+K^{191}, but modest dominant negative actions as C-tail pieces are added, particularly the F2 and F3 fragments (Fig. 6).

Discussion

The mammalian GnRHR is among the smallest of the G-protein-coupled receptors (327 amino acids in rodents and 328 amino acids in primates), owing, in part, to the absence of the long carboxyl terminal tail that is usually associated with members of this superfamily and is frequently involved with the development of the refractory state *(37)*. Because pre-mammalian GnRHRs contain long carboxyl terminal tails, its evolutionary truncation makes the mammalian GnRHR an attractive model for examining the role of this carboxyl terminal tail. It appears that this evolutionary truncation is associated with the restriction of plasma membrane expression and may reflect regulation needed for the added complexity of reproduction in mammals (i.e., cyclicity, two differentially regulated gonadotropins, modulation by sex steroids and peptide factors) *(38)*. On the other hand, the presence of the K^{191} in primate GnRHR further restricts PME by forming a metabolically unstable receptor that may potentially be recognized by the cellular quality control apparatus and degraded *(9,10)*. In fact, the

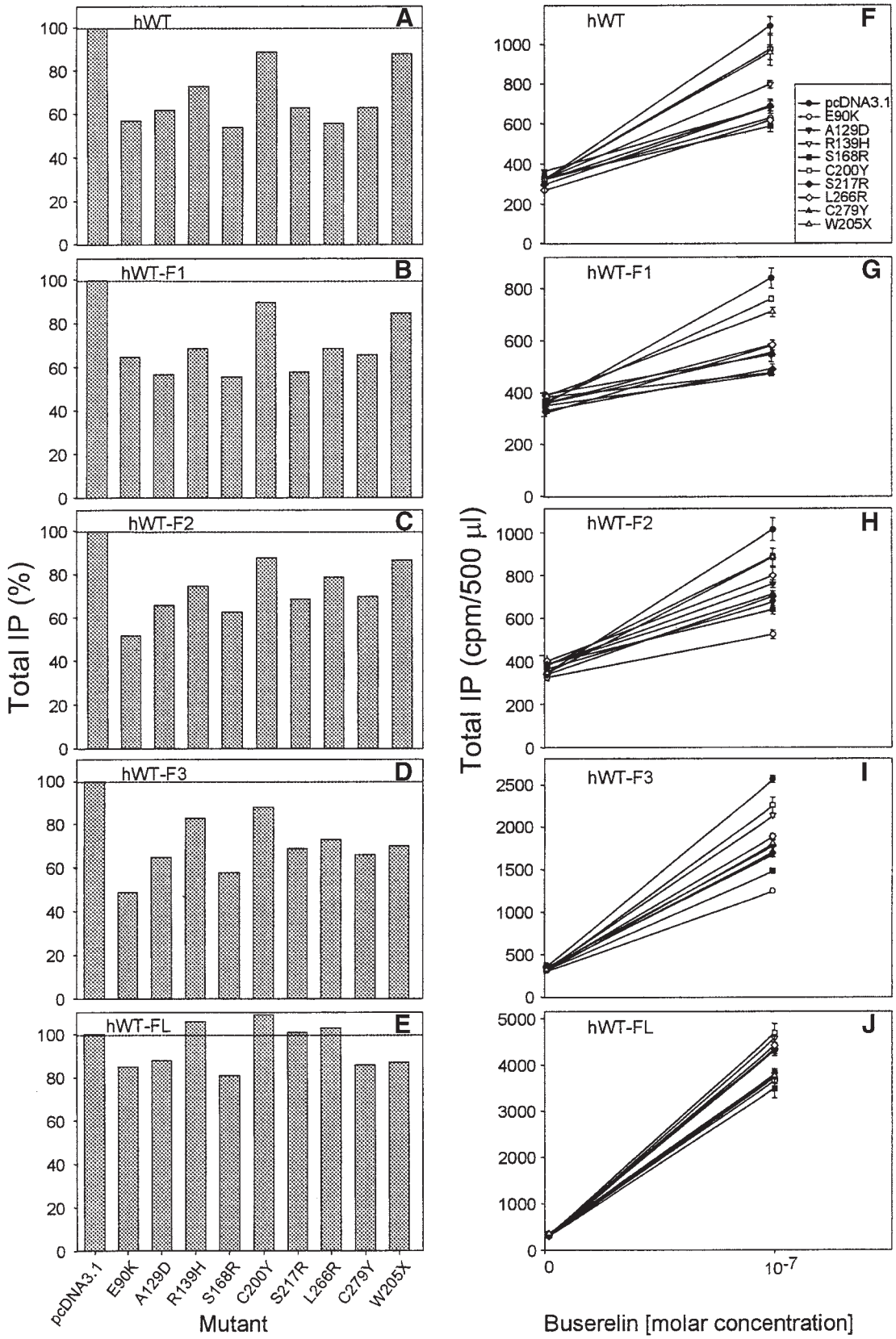

Fig. 3. Inhibition of (10^{-7} *M*) buserelin-stimulated IP production by co-expression of the different hGnRHR mutants with the human wt receptor at a mutant:wt DNA ratio of 8:1. Images on the right side are cpm and images on the left side are % control (the value for pcDNA3.1 with either wt or genetically modified receptor is 100%). ***a*** and ***f***, no C-tail added; ***b*** and ***g***, chimera with the shortest fragment, F3; ***c*** and ***h,*** chimera with F2; ***d*** and ***i,*** chimera with F1; ***e*** and ***j,*** chimera with the full length C-tail, FL. A horizontal dashed line is set at 100% IP3 production to allow for a better comparison between bar graphs. Data show the means ± SEM from at least two independent experiments each with triplicate incubations. pcDNA3.1 vector was used to keep the total amount of cDNA transfected constant.

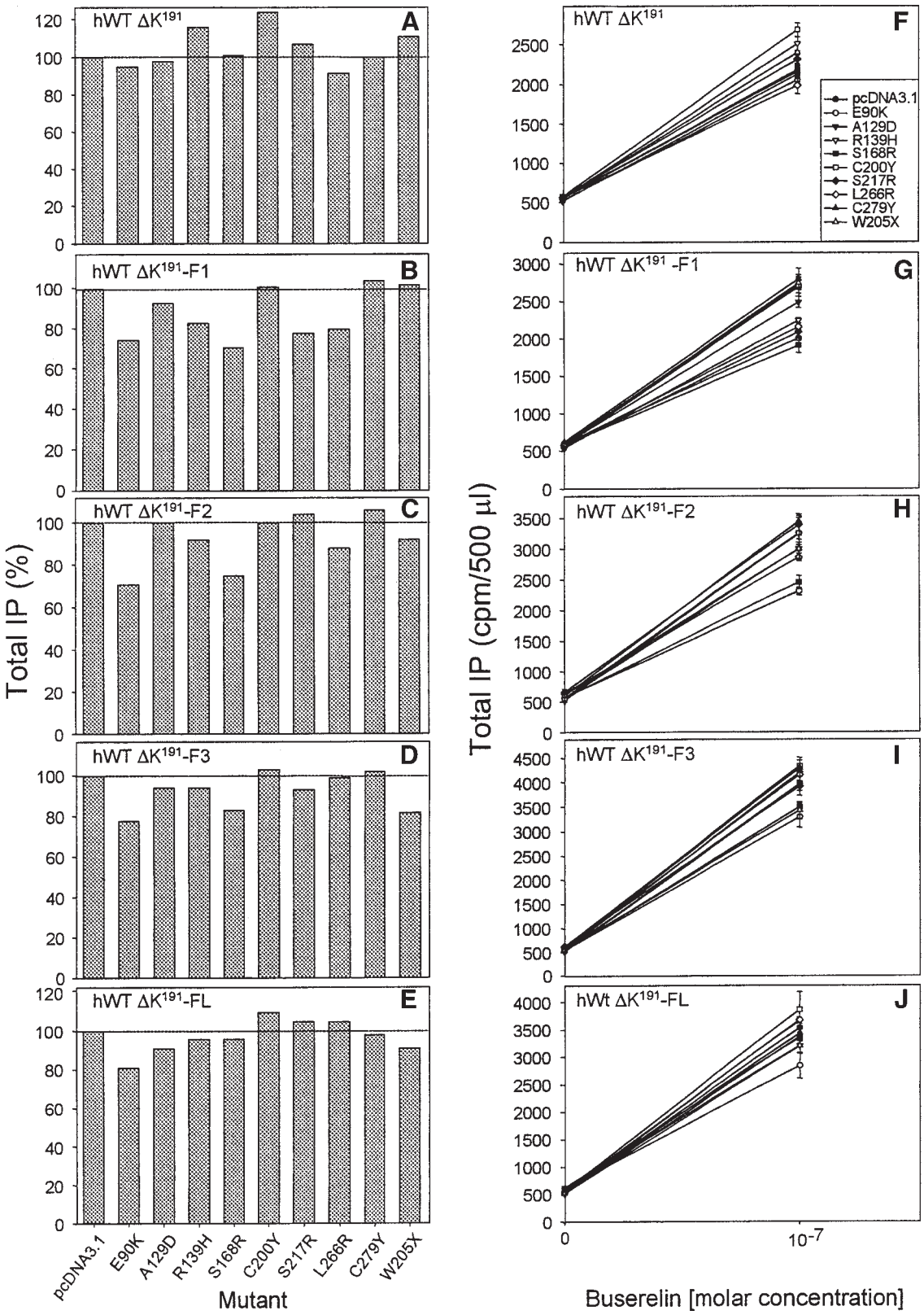

Fig. 4. Inhibition of (10^{-7} *M*) buserelin-stimulated IP production by co-expression of the different hGnRHR mutants with the human wt receptor lacking K^{191} at a mutant:wt DNA ratio of 8:1. Images on the right side are cpm and images on the left side are % control (the value for pcDNA with either wt or genetically modified receptor is 100%). ***a*** and ***f***, no C-tail added; ***b*** and ***g***, chimera with the shortest fragment, F3; ***c*** and ***h,*** chimera with F2; ***d*** and ***i,*** chimera with F1; ***e*** and ***j,*** chimera with the full length C-tail, FL. A horizontal dashed line is set at 100% IP3 production to allow for a better comparison between bar graphs. Data show the means ± SEM from at least two independent experiments each with triplicate incubations. pcDNA3.1 vector was used to keep the total amount of cDNA transfected constant.

pharmacoperone IN3, which stabilizes hGnRHR and thereby increases the efficiency of transit to the plasma membrane *(7,8)*, considerably improves the function of the wt hGnRHR, but not the wt rGnRHR, presumably by elevating the PME of the receptor protein. The ability to double the expression of hGnRHR at the plasma membrane with efficient pharmacologic chaperones (*7*, and present study) suggests that about half of the synthesized receptor never arrives at the plasma membrane and is eventually degraded.

The present data, which uses IP production to assess GnRHR receptor–effector coupling, indicates that (a) progressive addition of sections of the C-tail to the hwt receptor results in increased receptor–effector coupling efficiency and progressively blunted dominant-negative effects of the mutants, with a similar order of potency among the mutants; (b) removal of K^{191} from the hwt receptor results in increased PME and more modest negative effects of the mutants when the C-tail is progressively added, effects that eventually disappear; (c) addition of K^{191} to the rwt failed to restore dominant-negative effects of the mutants (or reduce expression to the level of the hwt GnRHR) in spite of a 88% human–rat GnRHR structural homology *(1,3)*. This suggests co-evolution of these two effects and requirement of other factors both for the dominant negative action of the mutant hGnRHRs and for decreased PME of the hwt receptor species, because removal of the C-tail and addition of K^{191} alone are not sufficient for the dominant negative effect of the mutants. The observation of limited expression of the hGnRHR is not unique to this protein. The importance of the cell's routing machinery in limiting expression of newly synthesized proteins is increasingly recognized *(9)*, and in fact, in many cells, 30–60% of these proteins never attain their correct native structure and are targeted for degradation *(39,40)*. The human δ-opioid receptor, for example, normally expresses as a low-efficiency endoplasmic reticulum (ER) export, with only approx 40% of the receptor reaching the cell surface *(39,40)*. Among the possibilities to consider as a potential third means of regulation of PME is glycosylation, because the human and rodent GnRHRs differ in the location of glycosylation sites. In hGnRHR, asparagine at position 18 glycosylates, while asparagines at positions 4 and 18 glycosylate in the rGnRHR *(41)*. The native GnRHR in pituitary membrane migrates on gels as a diffuse band *(42)*, characteristic of glycosylated proteins and this post-translational modification is known to be involved in membrane expression and/or stability of some receptors *(41,43–45)*.

In co-expression experiments, receptor binding and responses to agonist stimulation are frequently inversely proportional to the quantity of mutant cDNA co-transfected with the wt receptor. This effect has also been observed with V_2-vasopressin *(17)*, hGnRH *(21)*, and D2 dopamine *(46)* truncated receptor proteins, suggesting that co-existence of mutant and wt receptors may yield multimeric complexes that are impeded from attaining a conformation consistent with cell-surface transport. Although the precise mechanism(s) of the intermolecular interactions between GPCRs are unknown, it has been proposed that association between receptors may occur in the membranes of the ER during the process of specific interhelical interactions that lead to tight α-helical packing *(47)*. In this regard, it was striking to find that removal of a single amino acid (K^{191}) from the hGnRHR led to a dramatic increase in receptor function and reduction of the dominant-negative phenotype of the mutants. Nonetheless, addition of this basic amino acid residue alone was not sufficient to evoke dominant-negative effects of the mutants on rGnRHR.

It was interesting to observe that in addition to their negative effects on the hwt receptor and its F1–F3 chimeras, particular mutants exhibited dominant-positive effects on PME of some human (hwtΔK^{191}) and rat (rwt, rwt-FL, rwt+K^{191}, and rwt+K^{191}-F1) GnRH receptors. These findings suggest that conformational variants of receptors are prone to associate and form complexes, whose fate ultimately depends on the nature of the interaction between the receptors and the resulting conformation adopted by the particularly associated proteins. Although desirable to do so, we have been unable to use microscopic techniques to monitor the intracellular routing and membrane targeting of the GnRH receptors because the use of green fluorescent protein derivatives of this particular receptor *(48)* requires the presence of a catfish tail spacer *(26)*, which, itself, significantly influences receptor routing. Recent observations in our laboratory *(49)* also indicate that even short sequences required for HA-tagging rescues particular conformationally defective GnRHR mutants.

In hypogonadotropic hypogonadism due to GnRH resistance, affected individuals are either compound heterozygous or homozygous for the GnRHR mutation. Carriers of a mutant allele usually exhibit normal responsiveness to exogenous GnRH stimulation as well as normal gonadotropin levels and reproductive competence *(32,50)*. It is possible that these carriers express both the wt and the mutant receptor proteins at levels (e.g., 1:1 hGnRH mutant to wt hGnRHR ratios) compatible with expression of a normal phenotype. Alternatively, the expression levels of wt hGnRHR, albeit reduced by the negative effect of the mutant receptor, may otherwise be sufficient to mediate physiological effects.

In summary, it appears that a significant fraction of the wt hGnRHR is incompletely processed to the cell-surface membrane. Co-expression of hGnRHR mutants bearing folding defects may further aggravate the intrinsic functional deficit of the suboptimally expressed wt receptor population, probably owing to the formation of heterocomplexes that cannot escape the cellular quality control apparatus. Defective intracellular transport or interference with proper maturation owing to formation of misfolded complexes between the receptor species appears to explain the observed dominant-negative effect of the mutant hGnRH

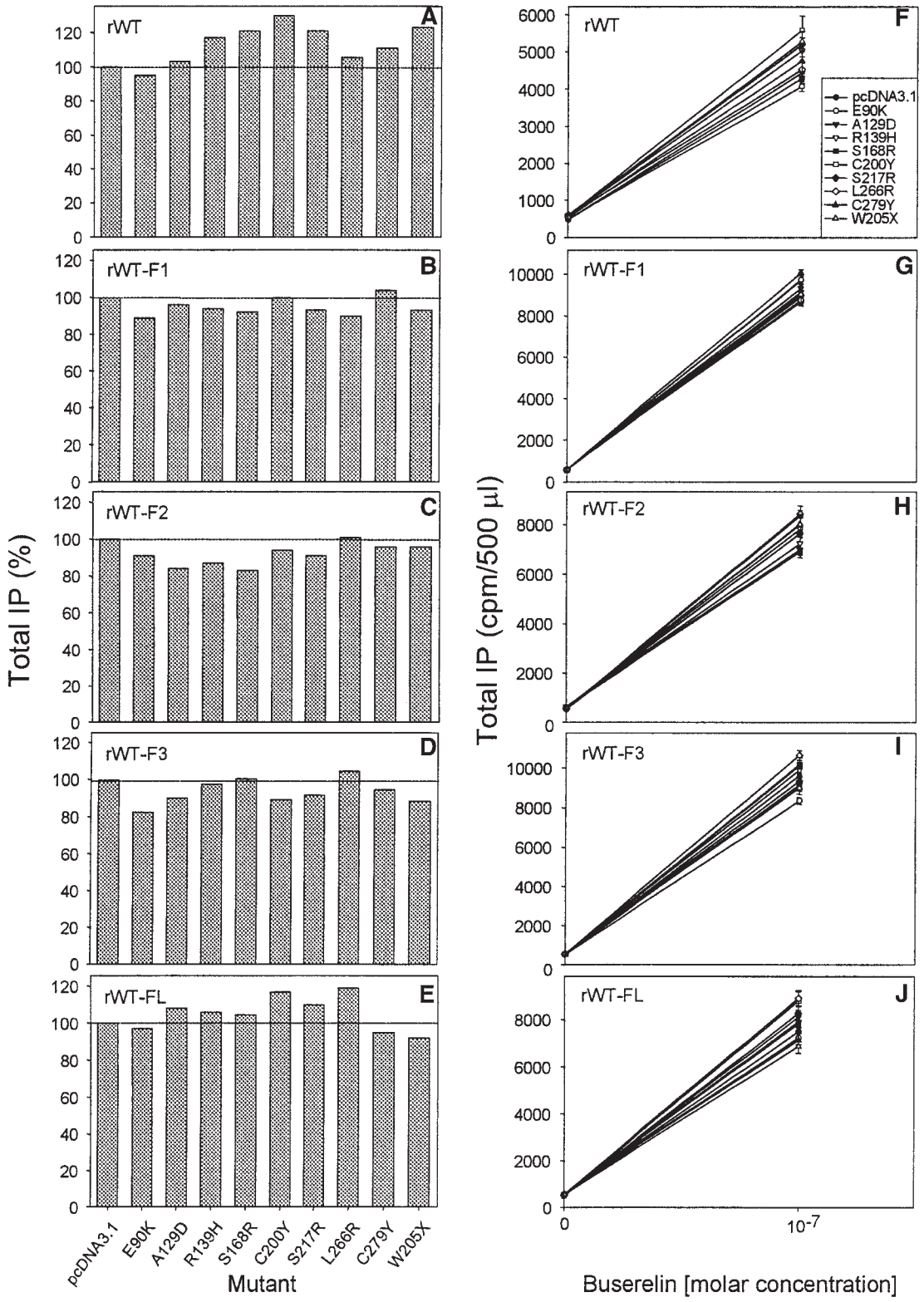

Fig. 5. Inhibition of (10^{-7} *M*) buserelin-stimulated IP production by co-expression of the different hGnRHR mutants with the rat wt receptor, at a mutant:wt DNA ratio of 8:1. Images on the right side are cpm and images on the left side are % control (the value for pcDNA with either wt or genetically modified receptor is 100%). ***a*** and ***f***, no C-tail added; ***b*** and ***g***, chimera with the shortest fragment, F3; ***c*** and ***h,*** chimera with F2; ***d*** and ***i,*** chimera with F1; ***e*** and ***j,*** chimera with the full length C-tail, FL. A horizontal dashed line is set at 100% IP3 production to allow for a better comparison between bar graphs. Data show the means ± SEM from at least two independent experiments each with triplicate incubations. pcDNA3.1 vector was used to keep the total amount of cDNA transfected constant.

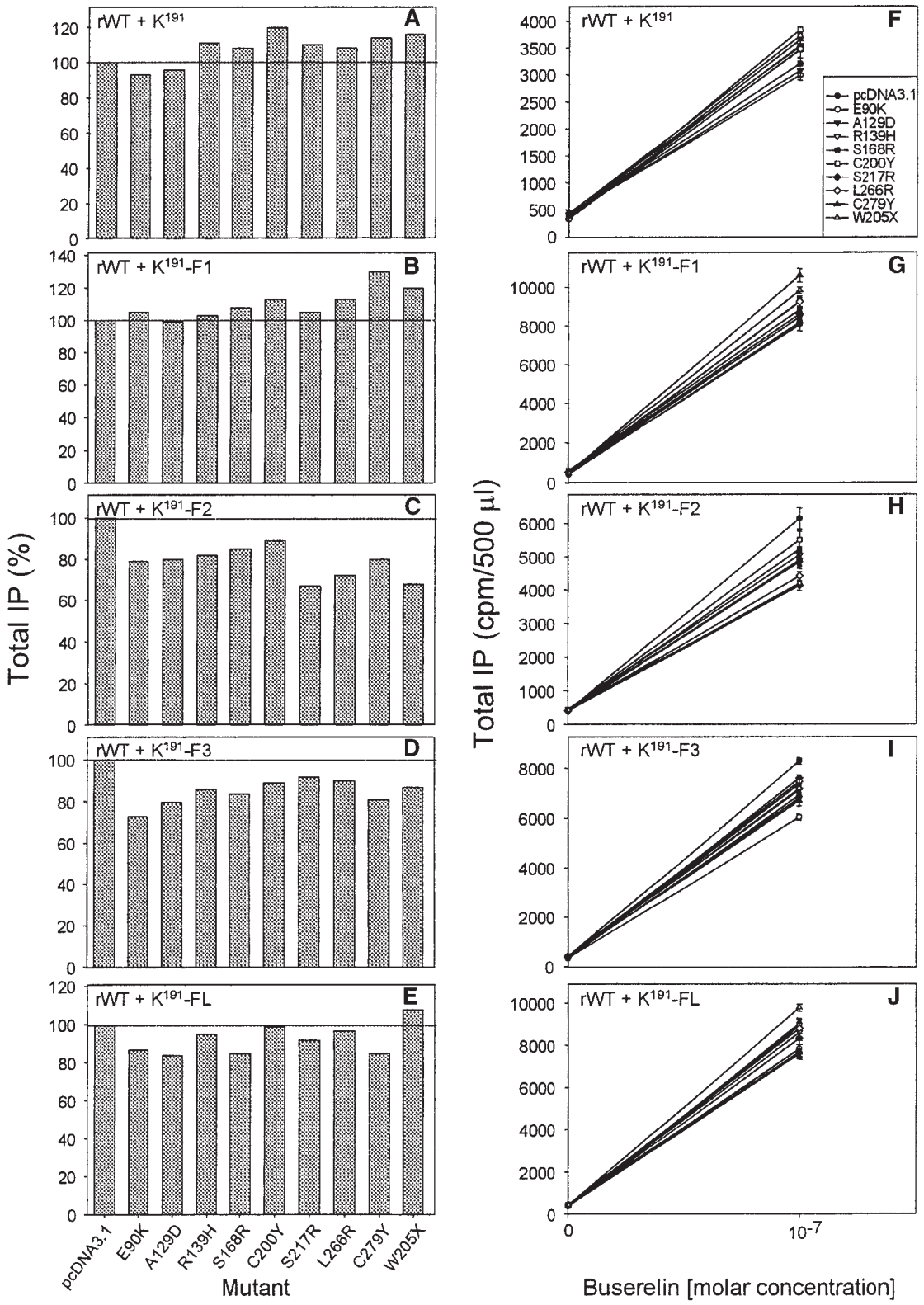

Fig. 6. Inhibition of (10^{-7} *M*) buserelin-stimulated IP production by co-expression of the different hGnRHR mutants with the rat wt receptor containing an added K^{191}, at a mutant:wt DNA ratio of 8:1. Images on the right side are cpm and images on the left side are % control (the value for pcDNA with either wt or genetically modified receptor is 100%). ***a*** and ***f***, no C-tail added; ***b*** and ***g***, chimera with the shortest fragment, F3; ***c*** and ***h,*** chimera with F2; ***d*** and ***i,*** chimera with F1; ***e*** and ***j,*** chimera with the full length C-tail, FL. An horizontal dashed line is set at 100% IP3 production to allow for a better comparison between bar graphs. Data show the means ± SEM from at least two independent experiments each with triplicate incubations. pcDNA3.1 vector was used to keep the total amount of cDNA transfected constant.

receptors. The unexpected finding of dominant positive effects of particular mutants with the hGnRHRΔK[191] or rat receptors may reflect enhanced chaperoning actions of highly expressed receptor forms. This may also explain why addition of the C-tail counteracts the negative actions of the mutant in the human, but not rat receptors. Conceivably, the (primate-derived) cell line used in this study has a quality control system that cannot recognize premammalian motifs for degradation and, thereby, enhances the PME. The data concurrently suggest that decreased PME is requisite for the dominant-negative effect of the hGnRH mutants, yet transfer of the K[191] to rGnRHR is alone insufficient to reduce the PME well enough to sensitize the receptor for the dominant-negative effect. It would be interesting to determine whether other wild-type GPCRs sensitive to negative or positive regulation by mutant congeners also exhibit intrinsically low maturation efficiencies and reduced expression, as documented here for the hGnRHR and previously for the δ-opioid receptors *(40,51)*.

Materials and Methods

Materials

Natural sequence GnRH was provided by the NIDDK National Hormone and Peptide Program (Bethesda, MD). The GnRH agonist, buserelin (D-tert-butyl-Ser[6], des-Gly[10], Pro[9], ethylamide-GnRH), was a kind gift of Hoeschst-Roussel Pharmaceutical (Somerville, NJ). The GnRH antagonists (used here as pharmacologic chaperones) IN3 and Q89 (Merck Research Laboratories) and A22509 (Abbott Laboratories) were provided as noted. The expression vector pcDNA3.1, Dulbecco's modified Eagle's medium (DMEM), OPTI-MEM, Lipofectamine, and PCR reagents were purchased from Invitrogen (Carlsbad, CA). Restriction enzymes, modified enzymes and competent cells for cloning were purchased from Promega (Madison, WI). Other reagents were of the highest degree of purity available from commercial sources.

Receptor Construction

Wild-type hGnRHR cDNA in pcDNA3 was subcloned into pcDNA3.1 at *Kpn*I and *Xba*I restriction enzymes sites. All naturally occurring hGnRHR mutants were constructed by overlap extension PCR *(52)* and sequence confirmed as previously described *(6)*. A truncated hGnRHR mutant was created by substituting a stop codon instead of the codon for amino acid 205 (*W205X*) using overlap extension PCR. Carboxyl terminal extensions were created as described previously *(26)* and sequences confirmed. We made similar chimeric constructs for human and rat GnRHR, each with or without the primate-specific K[191]. The full-length C-tail is a **S**er-rich (9 of 51 amino acid) sequence containing a consensus site for palmitoylation (*CXC*): **TPS**FRADL**S–**R***CFC***WR–NQNAS**AK–**S**LPHF**S**GHRREV**S**GEAE**S**DL G**S**GDQP**S**GQ, added to the C-terminal of the 328 amino acid sequence of the hwt receptor (327 amino acids for rwt receptor species) for a total of 379 residues. The truncation positions (dash marks) after positions correspond to 337 (F1), 343 (F2), 350 (F3), or 379 (full-length C-tail, FL) in the resultant chimera (Fig. 1).

Transient Transfection of COS-7 Cells

Wild-type hGnRHR, rGnRHR, and mutant receptors were transiently co-expressed in COS-7 cells as reported *(6)*. One hundred thousand cells/well were plated in 24-well plates (Costar, Cambridge, MA). Twenty-four hours later, the cells were co-transfected with hGnRHR mutant cDNAs (0.025 μg per well) and human or rat wt GnRHRs, human or rat GnRHR-cfC-tail chimeras, or hGnRHRΔK[191] or rat+K[191] (3.125 ng DNA per well) DNA constructs, as indicated, using 2 μL Lipofectamine in 0.25 mL OPTI-MEM. The total amount of DNA transfected remained constant as complementary amounts of the empty expression vector, pcDNA3.1, were included in the transfection mixture. After 5 h, 0.25 mL of DMEM containing 20% FCS was added to each well. The cells were incubated for an additional 18 h at 37°C. The transfection medium was removed and fresh growth medium was added to the cells for another 4 h at 37°C. The cells were then washed twice with DMEM/0.1% BSA/gentamicin and preloaded during 18 h with [^{3}H]myo-inositol for IP assays, as described below.

For experiments with the cell-permeant GnRH antagonists, IN3 [Merck Research Laboratories, Rahway, NJ, (2*S*)-2-[5-[2-(2-azabicyclo[2.2.2]oct-2-yl)-1,1-dimethyl-2-oxoethyl]-2-(3,5-dimethylphenyl)-1*H*-indol-3-yl]-*N*-(2-pyridin-4-ylethyl)propan-1-amine], Q89 [(7-chloro-2-oxo-4-{2-[(2*S*)-piperidin-2-yl]ethoxy}-*N*-pyrimidin-4-yl-3(3,4,5-trimethylphenyl)-1,2-dihydroquinoline-6-carboxamide); Merck compounds synthesized by Drs. Wallace T. Ashton and Mark Goulet, Merck Research Laboratories *(53)*, Rahway, NJ], and A222509, (3',3'-N-desmethyl-3',3'-N-cyclopropylmethyl-11-deoxy-11-[carboxy-(3-chloro,4-fluoro-phenylethylamino)]-6-O-methyl-erythromycin A 11, 12-(cyclic carbamate), Abbott Laboratories, N. Chicago, IL) were used at 1 or 2.5 μg/mL as indicated *(36)*. These structures were selected because of their predicted ability to permeate the cell membrane and interact with a defined affinity with the GnRHR; the three peptidomimetics have been shown to exhibit measurable efficacy in rescuing to different extents membrane expression and function of wt hGnRHR as well as a number of naturally occurring hGnRHR mutants *(7,8,36)*. Cultured COS-7 cells were transiently transfected with GnRHR cDNA [solutions containing either 1% DMSO (vehicle) or 1 μg/mL IN3, 2.5 μg/mL Q89, or 1 μg/mL A222509 prepared in the vehicle], as described *(36)*. Cells were continuously exposed to the antagonist during the period of transfection and thereafter until the start of the [^{3}H]myo-inositol preloading period.

Measurement of Inositol Phosphates (IP) Production

Quantification of IP production was performed by Dowex anion exchange chromatography and liquid scintillation spectroscopy, as described previously *(54)*.

Statistical Analysis

The data shown are the means ± SEM from triplicate IP determinations. In all experiments, the standard deviation was typically less than 10% of the corresponding mean, except at basal levels for which the cpm were extremely low. Each experiment was repeated two or more times with similar results; unless specified, the results of a single experiment are shown.

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