

G Protein-Coupled Receptor Trafficking in Health and Disease: Lessons Learned to Prepare for Therapeutic Mutant Rescue in Vivo

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Abstract—G protein-coupled receptors (GPCR) of receptor to convert external and internal stimuli to intracellular responses. As is the case with other membrane proteins, GPCRs are subjected to a stringent

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quality control mechanism at the endoplasmic reticulum, which ensures that only correctly folded proteins enter the secretory pathway. Because of this quality control system, point mutations resulting in protein sequence variations may result in the production of misfolded and disease-causing proteins that are unable to reach their functional destinations in the cell. There is now a wealth of information demonstrating the functional rescue of misfolded mutant receptors by small nonpeptide molecules originally designed to serve as receptor antagonists; these small molecules (“pharmacoperones”) serve as molecular templates, promoting correct folding and allowing the mutants to pass the scrutiny of the cellular quality control system and

be expressed at the cell surface membrane. Two of these systems are especially well characterized: the gonadotropin-releasing hormone and the vasopressin type 2 receptors, which play important roles in regulating reproduction and water homeostasis, respectively. Mutations in these receptors can lead to well defined diseases that are recognized as being caused by receptor misfolding that may potentially be amenable to treatment with pharmacoperones. This review is focused on protein misfolding and misrouting related to various disease states, with special emphasis on these two receptors, which have proved to be of value for development of drugs potentially useful in regulating GPCR trafficking in healthy and disease states.

I. Introduction

Synthesis and processing of proteins are tightly regulated events controlled at the transcriptional, translational, and post-translational levels. As proteins are synthesized in the endoplasmic reticulum (ER¹), they fold and adopt distinct conformations that are compatible with export to the Golgi apparatus for further processing (Radford and Dobson, 1999; Sitia and Braakman, 2003). Protein folding is a complex process not only because of the proximity and diversity of proteins that are synthesized but also because the steric character of the nascent protein backbone restricts the spectrum of shapes that may be recognized by a stringent quality control system (QCS). The QCS protects against aberrant cellular activity from misfolded molecules by monitoring protein folding and accumulation (Sanders and Nagy, 2000; Ellgaard and Helenius, 2001; Sitia and Braakman, 2003; Ulloa-Aguirre et al., 2004a). This QCS involves the participation of accessory components known as chaperones. Chaperones are a heterogeneous class of proteins that promote and facilitate folding and assembly. They do so by engaging in association with nascent proteins displaying particular features, such as the unexpected presentation of a hydrophobic plate in an aqueous environment, for example. This is important to prevent aggregation and/or interactions of misfolded proteins with other molecules present in a crowded ER environment and to assist in protein targeting to the Golgi complex or to its final destination within the cell (Hartl and Hayer-Hartl, 2002; Horwich, 2002). If chaperone-assisted protein folding fails, the conformationally defective protein is then targeted for degradation through the polyubiq-

uitination/proteasome pathway. Alternatively, misfolded proteins may aggregate, leading to potentially toxic intracellular accumulation or even to excessive protein accumulation in the plasma with extracellular amyloid deposition (Dobson, 1999; Kopito and Ron, 2000; Forloni et al., 2002; Chiti and Dobson, 2006). Thus, the ER QCS represents a potential site for therapeutic intervention in an array of diseases characterized by conformational aberrations of proteins.

It is becoming well recognized that mutations of receptors, enzymes, and ion channels frequently result in protein misfolding and subsequent retention by the cell's QCS (Tamarappoo and Verkman 1998; Burrows et al., 2000; Janovick et al., 2002; Leños-Miranda et al., 2002; Ulloa-Aguirre et al., 2003, 2004a,b; Bernier et al., 2004a,b; Ishii et al., 2004; Conn and Janovick, 2005; Loo et al., 2005; Yam et al., 2005; Pastores and Barnett, 2005; Suzuki, 2006; Ulloa-Aguirre and Conn, 2006; Wang et al., 2006). Misfolding can result in protein molecules that *retain* intrinsic function yet become misrouted within the cell and, *for reasons of mislocation only*, cease to function normally and result in disease. This observation contrasts with the prior presumption that mutational inactivation *always* reflects loss of intrinsic function (i.e., a receptor that either fails to recognize a ligand or does not couple productively to its effector). Recognition of this alternate concept immediately presents the therapeutic opportunity to correct misrouting and rescue mutants, thereby restoring function and, potentially, curing disease.

The importance of G-protein coupled receptor (GPCR) trafficking and cell surface membrane expression is emphasized by the array of diseases caused by receptor misfolding (Table 1). This is the case for the autosomal dominant forms of retinitis pigmentosa, X-linked nephrogenic diabetes insipidus, and hypogonadotropic hypogonadism (HH). The functional characterization of mutants that cause retinitis pigmentosa due to ER trapping of misfolded mutant rhodopsin and that eventually lead to photoreceptor degeneration was initially described by Sung et al. (1991, 1993) and thereafter by Kaushal and

¹ Abbreviations: ER, endoplasmic reticulum; QCS, quality control system; GPCR, G protein-coupled receptor; HH, hypogonadotropic hypogonadism; V2R, vasopressin type 2 receptor; GnRH, gonadotropin hormone-releasing hormone; GnRHR, gonadotropin hormone-releasing hormone receptor; LH, lutropin (luteinizing hormone); FSH, follicle stimulating hormone; DN, dominant-negative; PME plasma membrane expression; PM, plasma membrane; WT, wild type; UPR, unfolded protein response; TM, transmembrane; ECL, extracellular loop; si, small-interfering.

TABLE 1
Loss-of-function diseases or abnormalities caused by particular GPCR misfolding

The approximate number of total mutations reported, number of sites involved in the mutations and number of traffic-defective mutant receptors, and pharmacological chaperones used in *in vitro* studies are indicated. Information condensed as follows: rhodopsin (Stojanovic and Hwa, 2002; Mendes et al., 2005; Tao, 2006); V2R (Bernier et al., 2004a,b; Fujiwara and Bichet, 2005; Bichet, 2006; Boson et al., 2006; Robben and Deen, 2007; Robben et al., 2006); GnRHR (Beranova et al., 2001; Janovick et al., 2003a; Ulloa-Aguirre et al., 2004a,b); CaR (Brown, 2007; Huang and Breitwieser, 2007); LHR (Gromoll et al., 2002; Martens et al., 2002; Huhtaniemi and Themmen, 2005; Piersma et al., 2007); FSHR (Rannikko et al., 2002; Meduri et al., 2003; Huhtaniemi and Themmen, 2005); TSHR (Biebermann et al., 1997; Costagliola et al., 1999; Tonacchera et al., 2000, 2004; Calebiro et al., 2005; Davies et al., 2005); E-BR (Tanaka et al., 1998; Fuchs et al., 2001; Chen et al., 2006; Tao, 2006); MC1R-4R (Beaumont et al., 2005; Clark et al., 2005; Govaerts et al., 2005; Tao, 2005, 2006; Farooqi and O'Rahilly, 2006; Hinney et al., 2006; Lubrano-Bertheliet al., 2006; Alharbi et al., 2007; Lin et al., 2007); and CCR5 receptor (Lederman et al., 2006; Tao, 2006; Reiche et al., 2007).

| GPCR | Disease or Abnormality | Total No. of Mutations or Allelic Variants Reported | No. of Sites in the Receptor Compromised by Mutations | No. of Reported Traffic-Defective or PM Expression-Deficient Mutant Receptors | Pharmacoperones |
|--------------|--|---|---|---|--|
| Rhodopsin | Retinitis pigmentosa | 150 | 69 | 29 | 9- <i>cis</i> -retinal, 11- <i>cis</i> -7-ring retinal |
| V2R | Nephrogenic diabetes insipidus | 188 | 91 | 70 | SR121463 (satavaptan), SR49059 (relcovaptan), VPA-985, YM087, OPC41061 (tolvaptan), OPC31260 |
| GnRHR | Hypogonadotropic hypogonadism | 21 | 19 | 17 | Indoles, quinolones, erythromycin-derived macrolides |
| CaR | Familial hypocalciuric hypercalcemia | 61 | 50 | 9 | NPS R-568 |
| LHR | Male pseudohermaphroditism; hypergonadotropic hypogonadism | 25 | 20 | 9 | |
| FSHR | Ovarian dysgenesis | 9 | 9 | 7 | |
| TSHR | Congenital hypothyroidism | 32 | 28 | 8 | |
| E-BR | Hirschsprung's disease | 22 | 22 | 2 | |
| MC1R | Red head color and fair skin (RHC) phenotype and propensity to skin cancer | 61 ^a | 54 | 5 | |
| MC2R (ACTHR) | Familial glucocorticoid deficiency | 36 | 31 | Not tested | |
| MC3R | Obesity | 7 ^b | 4 | 1 | |
| MC4R | Obesity | 79 | 58 | 29 | |
| CCR5 | Resistance to HIV-1 infection | 19 | 19 | 4 | |

SR121463, satavaptan; SR49059, relcovaptan; VPA-985, 5-fluoro-2-methyl-*N*-[5*H*-pyrrol[2,1-*c*]-[1,4]benzodiazepin-10(11*H*)-ylcarbonyl]-3-chlorophenyl]benzamide; YM087, 4'-[(2-methyl-1,4,5,6-tetrahydroimidazo[4,5-*d*]1]benzazepin-6-yl)-carbonyl]-2-phenylbenzanilide monohydrochloride; OPC41061, tolvaptan; OPC31260, (\pm)-5-dimethylamino-1-[4-(2-methylbenzoylamino)benzoyl]-1,2,3,4,5-tetrahydro-1*H*-benzazepine monohydrochloride; CaR, calcium-sensing receptor; NPS R-568, *N*-(3-[2-chlorophenyl]propyl)-(*R*)- α -methyl-3-methoxybenzylamine; LHR, lutropin (luteinizing hormone) receptor; FSHR, follitropin (follicle-stimulating hormone) receptor; TSHR, thyrotropin receptor; E-BR, endothelin-B receptor; MC1R, melanocortin-1 receptor; MC2R, melanocortin-2 receptor [or adrenocorticotropin receptor(ACTHR)]; MC3R: melanocortin-3 receptor; MC4R, melanocortin-4 receptor; CCR5, chemokine receptor-5; HIV, human immunodeficiency virus.

^a Allelic variants *plus* mutations.

^b Allelic variants.

Khorana (1994). These reports were followed by descriptions of mutant vasopressin type 2 receptors (V2Rs), leading to nephrogenic diabetes insipidus caused by the inability of the mutant receptors to reach the cell surface membrane (Birnbaumer et al., 1994; Tsukaguchi et al., 1995; Wenkert et al., 1996; Sadeghi et al., 1997). More recently, mutations leading to receptor misfolding and resultant misrouting of the gonadotropin hormone-releasing hormone receptor (GnRHR) in patients with HH have been described previously (Janovick et al., 2002; Leños-Miranda et al., 2002; Ulloa-Aguirre et al., 2004b). There are other GPCRs in which mutations provoke loss of function of the receptor because of intracellular retention of the abnormal (and presumably misfolded and/or incompletely processed) receptor and, consequently, decreased or absent cell surface membrane expression. Some trafficking defective mutants of the glycoprotein hormone receptors [lutropin (LH), follitropin (FSH), and thyrotropin receptors] have been de-

scribed in patients with Leydig cell hypoplasia, a rare autosomal recessive form of male pseudohermaphroditism (LH receptor) (Gromoll et al., 2002; Martens et al., 2002), in women with ovarian dysgenesis (FSH receptor) (Rannikko et al., 2002; Meduri et al., 2003), and in congenital hypothyroidism (thyrotropin receptor) (Biebermann et al., 1997; Costagliola et al., 1999; Tonacchera et al., 2000, 2004). Loss-of-function mutations in the calcium-sensing receptor due to intracellular retention of the mutant receptor have been found in patients with familial hypocalciuric hypercalcemia (D'Souza-Li et al., 2002). The melanocortin-1 receptor, a major determinant for variations in skin and hair pigmentation, has been found to be mutated at different locations in patients with skin and hair abnormalities and increased susceptibility to skin cancers (Valverde et al., 1996); among the 60 or so mutants described, at least four display decreased cell surface expression (Beaumont et al., 2005). Intracellular retention of mutants from two other melanocortin-related receptors,

the melanocortin-3 and melanocortin-4 receptors, which are associated with regulation of fat deposition and energy homeostasis, respectively, have been detected in patients with morbid obesity (Ho and MacKenzie, 1999; Tao and Segaloff, 2003; Tao et al., 2006). Finally, mutations that lead to intracellular trapping in the ER of the endothelin-B receptor and the chemokine receptor 5 have been detected in patients with Hirschsprung's disease or aganglionic megacolon (Tanaka et al., 1998; Fuchs et al., 2001) and in subjects with resistance to human immunodeficiency virus infection (Rana et al., 1997), respectively. It is important to mention that in some cases, particularly in disease states with an autosomal recessive mode of inheritance, the defect in cell surface membrane expression is due to intracellular association of receptors, with a dominant-negative (DN) effect of the misfolded receptor on its wild-type counterpart (see section VI.); this DN effect may limit, or even abrogate, plasma membrane expression (PME) of the normal receptor and thus provoke a loss-of-function disease (Ulloa-Aguirre et al., 2004a). Concurrently, this information suggests that misfolding of GPCRs as a cause of disease may be actually substantially more common than previously recognized.

Pharmacological chaperones or "pharmacoperones" are small molecules that enter cells, bind specifically to misfolded mutant proteins, correct their folding, and allow them to escape retention by the cellular QCS (Sitia and Braakman, 2003; Schröder and Kaufman, 2005). In many cases, such molecules were initially identified as peptidomimetic antagonists selected from high throughput screens (although a priori, they need not be antagonists). For that reason they may come from diverse chemical classes. In the case of the GnRHR, pharmacoperones coming from classes as diverse as erythromycin macrolides, indoles, and quinolones have been identified (Janovick et al., 2003a).

Proteins rescued by pharmacoperones then route to the plasma membrane (PM) (or other site) where they can function normally. In principle, the pharmacoperone rescue approach might apply to an array of human diseases that result from misfolding, among these are cystic fibrosis (Dormer et al., 2001; Galiotta et al., 2001; Zhang et al., 2003; Amaral, 2006), HH (Ulloa-Aguirre et al., 2003), nephrogenic diabetes insipidus (Morello and Bichet, 2001; Bernier et al., 2004b; Bichet, 2006), retinitis pigmentosa (Noorwez et al., 2004), hypercholesterolemia, cataracts (Benedek et al., 1999), neurodegenerative diseases [Huntington's, Alzheimer's, and Parkinson's diseases (Heiser et al., 2000; Soto et al., 2000; Forloni et al., 2002; Permanne et al., 2002; Muchowski and Wacker, 2005)], and cancer (Peng et al., 2003). In the case of particular proteins (e.g., the GnRHR, the V2R, and rhodopsin), this approach has succeeded with a striking number of different mutants, supporting the view that pharmacoperones will become powerful ammunition in our therapeutic arsenal (Bernier et al., 2004b). On the other hand, it has also

become clear that variable amounts of even some WT GPCRs are misrouted, presumably as a result of misfolding (Petäjä-Repo et al., 2000, 2001; Andersson et al., 2003; Janovick et al., 2003b; Lu et al., 2003, 2004; Cook et al., 2003; Pietilä et al., 2005), suggesting that this level of post-translational control may itself be amenable to pharmacological intervention and provide another level of potential therapeutic intervention (Ulloa-Aguirre et al., 2006).

We have previously reviewed the literature on diseases associated with folding in general, all potential targets for pharmacoperone therapeutics (Castro-Fernández et al., 2005), and now focus on translation of this concept to in vivo models. In this review we specifically focus on what we have learned in cell culture studies that is likely to become useful for controlling trafficking of receptors, ion channels, and enzymes in healthy and disease states. We focus on opportunities for drug development and on the lessons learned from two well characterized models of GPCRs, the GnRHR (Ulloa-Aguirre et al., 2003, 2004a, 2006) and the V2R (Morello et al., 2000, 2001; Petäjä-Repo et al., 2002; Bernier et al., 2004a,b; Bulenger et al., 2005). Before describing the particular pharmacological approaches potentially applicable to misfolded GnRHR and V2R, let us briefly review how the ER QCS works to prevent normal routing of defective proteins.

II. Endoplasmic Reticulum Quality Control System and Molecular Chaperones

According to current models of protein folding, as proteins are synthesized in the ER, they fold and adopt a distinct conformation that allows the protein molecule to acquire a stable structure compatible with ER export (Radford and Dobson, 1999; Sanders and Nagy, 2000; Trombetta and Parodi, 2003). The ER QCS recognizes specific shapes resulting from protein wriggling and hence defines the routing, intracellular trafficking, and eventually the fate of the nascent protein within the cell (Ellgaard and Helenius, 2001; Cahill et al., 2002). To this end, the ER QCS employs a variety of mechanisms including a complex sorting system that identifies and separates proteins according to their maturation status and the action of specialized folding factors, escort proteins, retention factors, enzymes, and members of major molecular chaperone families. Molecular chaperones are accessory components of the ER QCS that participate in the folding process of newly synthesized proteins (Ellgaard and Helenius, 2001; Sitia and Braakman, 2003). They serve as a control mechanism recognizing, retaining, and targeting misfolded proteins for their eventual degradation. Although the steric character of the protein backbone restricts the spectrum of protein shapes that are recognized by the stringent quality control mechanisms, some features displayed by proteins including exposure of hydrophobic shapes, unpaired cysteines, or

immature glycans have been identified as important in chaperone-protein association (Ellgaard and Helenius, 2001); in fact, molecular chaperones possess the ability to recognize misfolded proteins by the exposure of hidden hydrophobic domains or particular motifs (Tan et al., 2004; Dong et al., 2007). Through this association, chaperones attempt to stabilize unstable conformers of nascent polypeptides to prevent aggregation and facilitate correct folding or assembly of the substrate via binding and release cycles (Hartl and Hayer-Hartl, 2002). If the polypeptide chain fails to fold properly, then the incorrectly manufactured protein is targeted to the proteasomes for destruction (Werner et al., 1996; Schubert et al., 2000). Several GPCR interacting proteins that support trafficking to the cell surface have been identified. Nina A (neither inactivation nor afterpotential A) is a molecular chaperone whose absence in *Drosophila melanogaster* rhodopsins leads to rhodopsin 1 ER accumulation and degradation (Schneuwly et al., 1989; Shieh et al., 1989; Colley et al., 1991; Baker et al., 1994); its mammalian homolog RanBP2 binds red/green opsin molecules and acts as a chaperone aiding proper folding, transport, and localization of the mature receptors to the cell membrane (Ferreira et al., 1996). ODR4 is a molecular chaperone that assists in folding, ER exit, and/or targeting of the olfactory receptors ODR10 to olfactory cilia in the nematode *Caenorhabditis elegans* (Dwyer et al., 1998). Calnexin and calreticulin are molecular chaperones that bind a broad range of glycoproteins, including several GPCRs (e.g., the GnRHR, V2R, and LH, FSH, and thyrotropin receptors) (Helenius et al., 1997; Schrag et al., 2003; Vassilakos et al., 1998; Rozell et al., 1998; Morello et al., 2001; Brothers et al., 2006). The action of these chaperones predominantly centers on substrate *N*-glycans present on the newly synthesized proteins, adding hydrophobicity to the folding protein (Helenius et al., 1997; Schrag et al., 2003). When *N*-glycosylation or early glycan processing fails (due to mutations in the glycosylation sites of the receptor, for example), glycoproteins misfold, aggregate, and fail the QCS. This is the case, for example, of the V2R R337X mutant, in which an extended interaction between calnexin and the mutant receptor is involved in ER retention and the absence of cell surface membrane expression of the mutant receptor (Morello et al., 2001). Other molecular chaperones that aid GPCRs to reach the cell surface membrane have been described. These include RAMPs (receptor activity modifying proteins), which interact with several GPCRs [e.g., the calcitonin receptor-like receptor, the vasoactive intestinal polypeptide/pituitary adenylate cyclase-activating peptide receptor, the glucagon receptor, and the parathyroid hormone receptor, fostering transport of the associated receptor to and regulating its signaling function at the PM (Christopoulos et al., 2003); gC1q-R, receptor for globular heads of C1q, which interacts with the carboxyl terminus of the α_{1B} -adrenergic receptor and regulates

the maturation and expression of the receptor (Xu et al., 1999); and BiP/Grp78, a chaperone involved in the protective unfolded protein response (UPR), which is a cell stress program activated when misfolded proteins accumulate in the lumen of the ER (Yang et al., 1998; Schröder and Kaufman, 2005)] (see paragraph below).

It is recognized that continuous ER stress, such as that provoked by the accumulation of unfolded proteins, results in cell death and relates to the pathogenesis of some neurodegenerative diseases (Forman et al., 2003). Accumulation and aggregation of misfolded proteins are presumably responsible for some neurodegenerative diseases such as early-onset familial Alzheimer's disease, Parkinson's disease, and prion disease (Forloni et al., 2002). In these diseases, the soluble conformations of proteins or fragments of proteins convert to insoluble fibrillar aggregates, known as amyloids, which are formed by cross- β -pleated sheet structures that accumulate intra- and/or extracellularly (Glennner, 1980; Dobson, 1999; Forloni et al., 2002). A set of interlinked molecular pathways, collectively referred to as the UPR, are activated by ER stress; overwhelming or failure of the UPR may lead to apoptosis and thus play an important role in the pathogenesis of the above-mentioned neurodegenerative disorders (Forman et al., 2003). Several quality control factors participate in the UPR, including the ER chaperone BiP/Grp78, which negatively regulates three proximal sensors, the transmembrane kinase and endoribonuclease IRE1, pancreatic ER kinase, and activating transcription factor 6 (Yang et al., 1998). When unfolding or misfolding occurs, BiP dissociates from the sensors and binds the unfolded proteins in an attempt to refold them; this dissociation releases the sensors from negative inhibition, leading to the activation of multiple signaling pathways and induction of UPR-inducible genes and decreased protein expression (Forman et al., 2003). These changes increase the folding capacity of the ER, reduce new protein translocation to the ER, and increase the degradation of the abnormally folded or unfolded proteins (Harding et al., 2002; Kaufman, 2002). Whereas prolonged UPR activation may lead to apoptosis, several proteins presumably involved in amyloid-forming disorders may promote or inhibit various steps in the UPR (Forman et al., 2003). Little attention has been paid to the potential role of the ER stress response on the pathogenesis of diseases caused by misfolding and intracellular accumulation of GPCRs, and there is no evidence to date that aggregation of misfolded GPCRs may follow the catastrophic fate observed for proteins that cause neurodegenerative diseases. Nevertheless, the observation that *in vitro* expression of the misfolded mutant Pro²³His of rhodopsin (the most frequent rhodopsin mutation leading to retinitis pigmentosa) results in formation of aggregates due to a generalized impairment of the ubiquitin-proteasome system (which is potentially toxic to the cell) (Saliba et al., 2002; Illing et al., 2002), strongly suggests the exist-

tence of a link between the mechanisms leading to photoreceptor degeneration in retinitis pigmentosa and those that participate in the genesis of neurodegenerative diseases, including the ER UPR.

III. Physiology of the Gonadotropin-Releasing Hormone Receptor and Vasopressin Type 2 Receptor Systems

A. The Human Gonadotropin-Releasing Hormone Receptor and Vasopressin Type 2 Receptor in Health and Disease

The mammalian GnRHR type I (hereafter referred as GnRHR) (Fig. 1A) belongs to the superfamily of G protein-coupled receptors, specifically to the family related to the rhodopsin and β -adrenergic receptors (family A). The GnRHR is located in the pituitary gonadotrope and is bathed by the circulation of the (closed) hypothalamic-pituitary portal system, which transfers pulsatile signals of the hypothalamic decapeptide, GnRH (shown). The gonadotrope cell responds with a concomitant pulsatile release of the gonadotropins, LH, and FSH (Santen and Bardin, 1973; Knobil, 1974). These enter the peripheral circulation and regulate gonadal steroidogenesis, along with maturation of eggs and sperm. Intermittent exposure of the GnRHR to the releasing hormone is important from a functional point of view; slower GnRH pulses favor release of FSH whereas faster pulses favor release of LH (Belchetz et al., 1978; Crowley et al., 1985; Hazum and Conn, 1988) (Fig. 2). Frequency modulated signals are also important to prevent desensitization (refractoriness) of the gonadotrope to a subsequent stimulus, allowing for the occurrence of distinct rates and patterns of synthesis and release of the gonadotropins that follow GnRH exposure (Belchetz et al., 1978). The GnRHR is among the smallest members of the GPCR superfamily (328 amino acid residues in the human GnRHR) and bears unique structural features, including the lack of a carboxyl-terminal intracellular tail (Millar et al., 2004). Fish, reptiles, birds, and the primate type II GnRHR (McArdle et al., 1999; Millar, 2003) do possess this carboxyl extension whose presence is associated with differential physiological receptor regulation (Lin et al., 1998); when added to the mammalian GnRHR, it dramatically increases PME levels of this receptor (Janovick et al., 2003b). Another important feature of the GnRHR is the amino acid residue in position 191, which is frequently Glu or Gly but is replaced by Lys in primates (Janovick et al., 2006); in rat and mouse GnRHR, this amino acid is absent (Arora et al., 1999). The GnRHR is coupled to the trimeric Gq/11 protein, whose activation stimulates the effector enzyme phospholipase-C β , leading to phosphatidylinositol 4,5-bisphosphate hydrolysis and formation of the second messengers, inositol 1,4,5-triphosphate and diacylglycerol (Conn et al., 1986). The former messenger diffuses through the cytoplasm, promoting the release of intra-

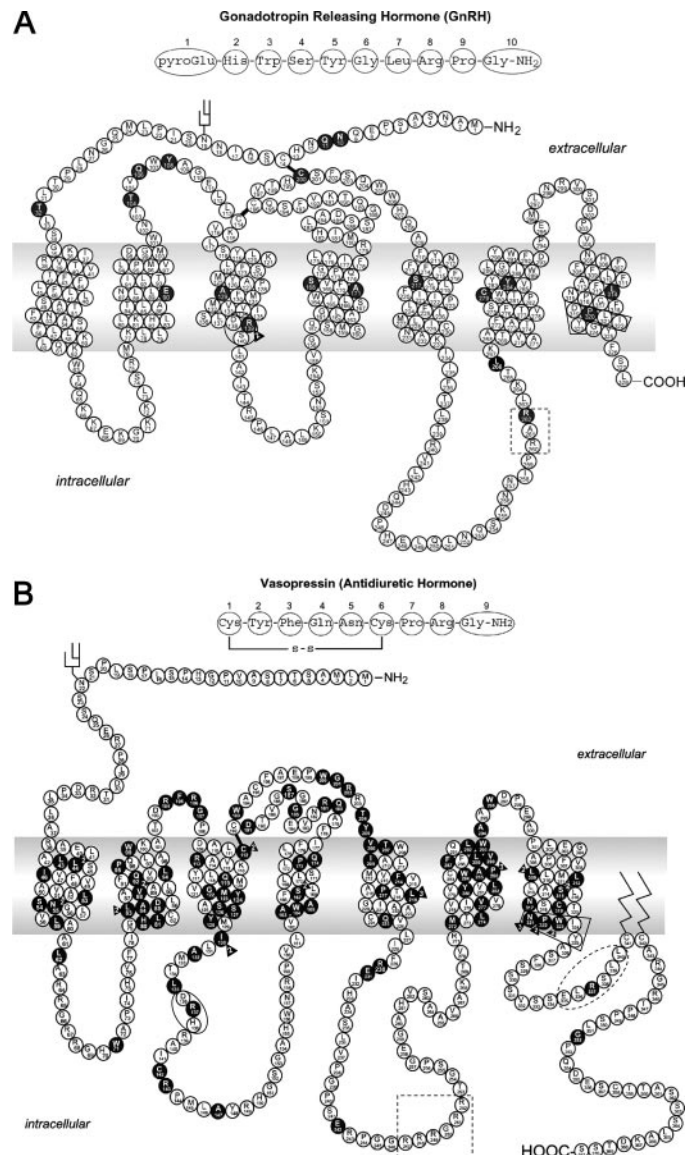


FIG. 1. Structures of the GnRHR (A) and V2R (B) showing their ligands and sites of mutations (dark circles) that are associated with human disease. When there are multiple mutations at a single site, the number of mutations is noted in a triangle. ER retention motifs are indicated with dashed line squares; the ER export motif E(X)₃LL in the carboxyl terminus of the V2R is indicated by a dashed-line oval. Also indicated are the DRY motif (permutated to DRS in the GnRHR and to DRH in the V2R) in the amino-terminal end of the second intracellular loop (solid line oval) and the D/NpXXY motif in the seventh transmembrane helix of both receptors (solid line polygons). Glycosylation sites are represented by branched structures.

cellular calcium and the release of both gonadotropins. More recently, coupling of the GnRHR to the cAMP pathway has been shown under conditions of sustained stimulation, which may be potentially important under intense GnRH release, such as the preovulatory GnRH surge (Larivière et al., 2007).

Gonadotropin-releasing hormone, the natural ligand of the GnRHR, interacts with several amino acid residues of the receptor located mainly in the transmembrane (TM) domains: these include Asp⁹⁸, Asn¹⁰², Asp³⁰², Trp¹⁰¹, Lys¹²¹, Asn²¹², and Tyr²⁹⁰; GnRH pep-

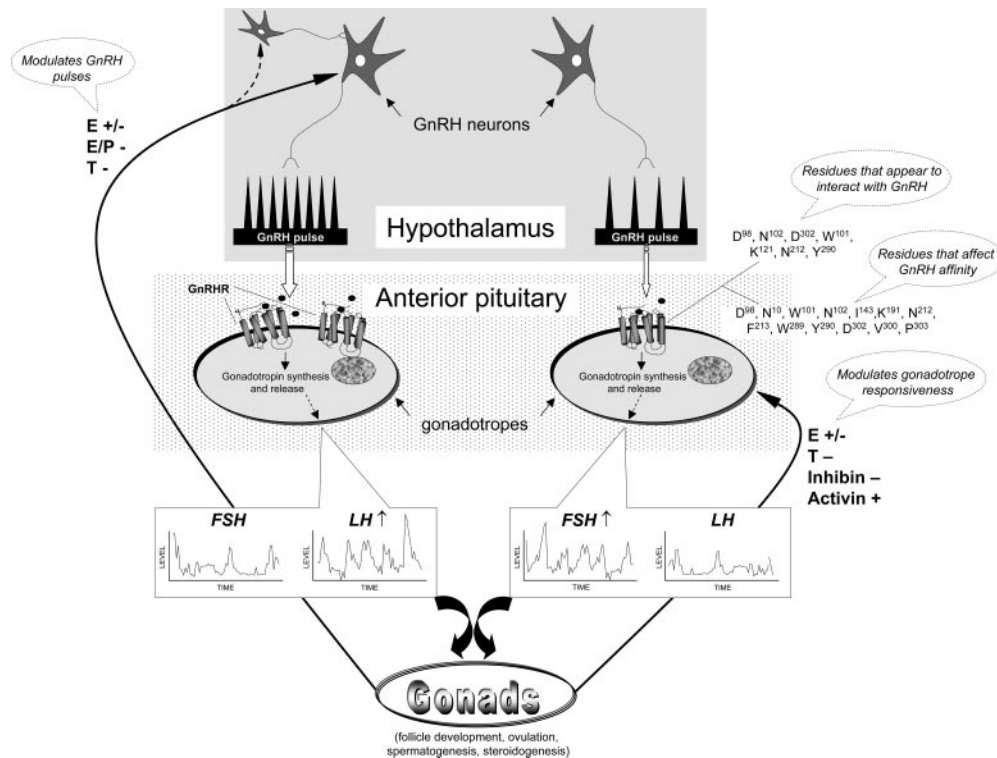


FIG. 2. Summary of gonadotropin hormone-releasing hormone frequency modulation of gonadotropin release and regulatory hormonal feedback loops in the hypothalamic-pituitary-gonadal axis. GnRH is synthesized and secreted by specialized neurons located in the arcuate nucleus of the medial basal hypothalamus and the preoptic area of the anterior hypothalamus. The secretion and interaction of GnRH with its cognate receptor occurs in a pulsatile and intermittent manner. The gonadotrope cell responds with resultant pulsatile release of the gonadotropins: LH is favored when GnRH pulse frequency is faster, whereas FSH is favored by a slower pulse frequency. Gonadotropins bind to specific GPCRs on the gonads to stimulate the production of sex steroids [17β -estradiol (E), progesterone (P), and testosterone (T)] and peptide hormones (inhibin and activin) that modulate GnRH and gonadotropin release. In the hypothalamus, E may act directly on GnRH neurons, but all steroids may act indirectly through other hypothalamic neurons that subsequently influence GnRH release (dashed arrow). Elevated T concentrations suppress (-) hypothalamic GnRH pulses and the pituitary response to GnRH. Estradiol treatment alone increases (+) GnRH pulse frequency but decreases pulse amplitude, whereas E plus P suppress GnRH pulses. At the pituitary level, E increases the number of GnRHRs and the responsiveness of LH genes to GnRH; it can also directly suppress LH secretion. GnRH also has a self-priming (up-regulation) and down-regulating action on its own receptor. Human GnRHR amino acid residues that regulate GnRH binding and affinity are also shown.

tide agonist binding sites overlap with some natural ligand binding sites, and also may interact with Trp²⁸⁰ and Phe²¹⁶, depending on the particular structure of the agonist (Sealfon et al., 1997; Millar et al., 2004). Peptide antagonists occupy GnRHR binding sites that differ from, but that may overlap, the agonist binding pocket, as suggested by mutational analysis and molecular dynamics simulations (Millar et al., 2004; Söderhall et al., 2005). Nevertheless, the fact that agonists, antagonists, and inverse agonists may exhibit distinct selectivities toward the active and the inactive conformation of the receptor suggests that competitive antagonism may occur without any overlap with agonist binding sites (Samama et al., 1993). Binding of nonpeptide antagonists is less known; nevertheless, studies on a few nonpeptide small, quinolone- and thienopyridine-based GnRH antagonists indicate that their binding sites partially overlap GnRHR residues important for GnRH binding (Cho et al., 1998; Cui et al., 2000); in addition, Phe³¹³ has been proposed as a site critical for the binding of this class of antagonists to the human GnRHR (Cui et al., 2000).

Loss-of-function mutations in the GnRHR can lead to partial or complete hypogonadotropic hypogonadism, a failure of pituitary gonadotropes to respond to GnRH, which results in decreased or apulsatile gonadotropin release and reproductive failure (Ulloa-Aguirre et al., 2004a). To date, 21 inactivating mutations (including two leading to missing of large sequences) in the human GnRHR gene have been described as a cause of HH (Figs. 1A and 3). Seven homozygous and 12 heterozygous combinations of human GnRHR mutants are expressed by individuals exhibiting either partial or complete forms of HH (Beranova et al., 2001; Ulloa-Aguirre et al., 2004b). The majority (~90%) of the GnRHR mutants whose function has been examined to date (17 mutants) are trafficking-defective receptors as disclosed by mutational studies and/or responses to pharmacoperones (see section III.C.). Because reproductive failure is not life-threatening, it is likely that many cases (particularly partial HH forms) go undiagnosed and, individual mutants, if severe in the phenotype, are not passed to progeny.

The V2R (Fig. 1B) is another family A member of the GPCR superfamily. In humans, the V2R comprises 371

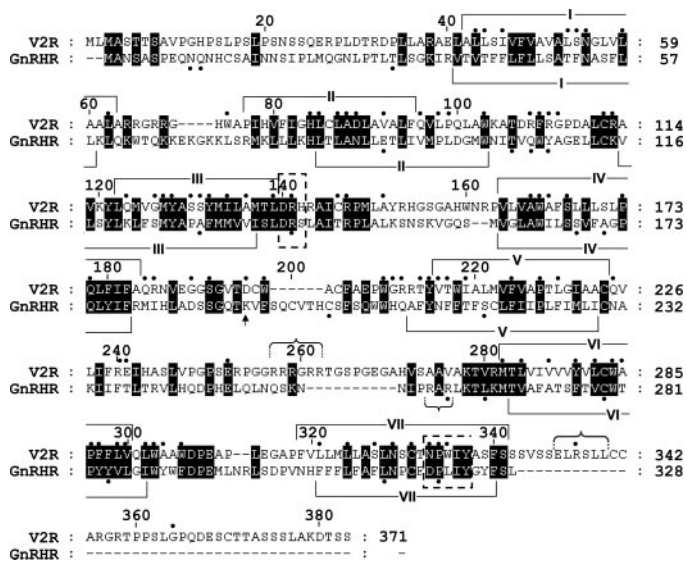


FIG. 3. Sequence alignment of the human GnRHR and the V2R. The limits of the transmembrane helices are indicated by brackets above (V2R) or below (GnRHR) sequences. The ER retention or export motifs are indicated by open keys and the DRY (permuted to DRH in the GnRHR and to DRH in the V2R) and N/DPxxY motifs by dashed line squares. Dots above (V2R) or below (GnRHR) sequences indicate the location of the mutations reported to date. Numbers above the sequence indicate the amino acid number of the aligned sequences. Numbers at the right of each lane indicate the amino acid number of nonaligned sequences.

amino acid residues, and the receptor is expressed and localized in the basolateral membrane principal cells of the renal collecting duct of the kidney. V2R normally binds the nonapeptide arginine-vasopressin (shown). Agonist occupancy of this receptor stimulates activation of the receptor-coupled G_s protein, activation of adenylyl cyclase, production of cAMP, and activation of protein kinase A. The phosphorylation started by this kinase promotes translocation and exocytic insertion of the specific water channel protein aquaporin-2 to the luminal membrane, resulting in water reabsorption in the kidney (Birnbauer, 2000, 2002). Agonists and antagonists (peptide and nonpeptide) seem to prefer a common V2R compartment for docking (Czaplewski et al., 1998a,b; [oe]lusarz et al., 2006). Molecular modeling of the V2R has revealed that the receptor amino acid residues potentially important in ligand binding are located mainly in TM domains 3 to 7, including Cys¹¹², Val¹¹⁵ and Lys¹¹⁶, Gln¹¹⁹, and Met¹²³ (helix 3), Glu¹⁷⁴ (helix 4), Val, 20⁶, Ala²¹⁰, Val²¹³, and Phe²¹⁴ (helix 5), Trp²⁸⁴, Phe²⁸⁷ and Phe²⁸⁸, and Gln²⁹¹ (helix 6), and Phe³⁰⁷, Leu³¹⁰, Ala³¹⁴, and Asn³¹⁷ (helix 7) (Czaplewski et al., 1998b).

Nearly 188 inactivating mutations of the V2R causing X-linked diabetes insipidus have been described previously (Fujiwara and Bichet, 2005; Bichet, 2006; Boson et al., 2006); the vast majority of these mutations correspond to the so-called type 2 V2R mutant receptor (Bichet, 2006; Morello and Bichet, 2001). Furthermore, 89 of these 184 mutations are missense mutations likely to result in misfolded proteins that are trapped in the

ER and are unable to reach the basolateral cell surface to engage the antidiuretic hormone arginine-vasopressin (Bichet, 2006). These defective receptors accumulate in different compartments of the early secretory pathway, depending on their folding state (Hermosilla et al., 2004). The urine of patients with diabetes insipidus is not concentrated in response to (the antidiuretic hormone) arginine-vasopressin. Accordingly, the lack of concentration results in severe dehydration in the absence of adequate replacement hydration and elevated sodium levels.

B. Lessons from Comparison of the Gonadotropin-Releasing Hormone Receptor and Vasopressin Type 2 Receptor Systems: Selecting Likely Targets for Rescue by Pharmacoperones

In considering these two systems, each with their own set of mutants (sites shown as dark circles in Fig. 1, A and B) and their own chemically distinct pharmacoperones (Janovick et al., 2003a; Bernier et al., 2004a), some commonalities that may provide important clues about how to identify systems that are especially amenable to this rescue approach are evident: 1) Both the GnRHR and the V2R recognize *small* peptide ligands, a decapeptide and nonapeptide sequence, respectively; 2) each ligand is only slightly larger than 1000 Da in mass (Fig. 1, A and B), and both peptide ligands are somewhat *hydrophobic* in nature; and 3) because these are small molecules, it is likely that the compartments for docking in their corresponding receptor are also relatively small, and both receptors are believed to bind ligand mainly in their TM sections, consonant with their hydrophobic nature (Czaplewski et al., 1998a,b; Millar et al., 2004). It is reasonable to assume that significant features such as the ligand binding site would be maintained during evolution, as the general structure of both ligands has been conserved. In fact, many of the residues involved in ligand binding are relatively invariant for the GPCR superfamily (Ulloa-Aguirre and Conn, 1996; Millar et al., 2004).

Both receptors themselves are also relatively *small*, compared with other GPCRs; as mentioned in section III.A., the human GnRHR is 328 amino acids and the human V2R is 371 amino acids. Sequence identity between the human GnRHR and the V2R is 20%, whereas their similitude (i.e., sharing similar residues or conservative substitutions) is 39% (Fig. 3). These results may simply reflect the conserved nature of the TM domains among GPCRs. The cytoplasmic extensions of the amino termini of both receptors are quite short, as are those of the carboxyl termini—the GnRHR has none at the carboxyl end. This protein actually terminates in the cytoplasmic face of the membrane. The intra- and extracellular loops are, as the total size would predict, quite small (Fig. 1, A and B). The general size similarities between these two receptors, taken as a whole, suggest that the “correctly” folded (that is, the structure that

passes the QCS) structures of small receptors with small ligands (i.e., small ligand binding site) might be quite sensitive to distortion and, accordingly, are easily recognized as defective by the QCS. This may explain why these two receptors, as well as human rhodopsin (348 amino acid residues), are the most frequently affected among the GPCR superfamily by mutations leading to ER trapping and disease (Tan et al., 2004). In addition, it is reasonable to consider the possibility that a mutation may have a proportionally larger effect on a small, compact structure than on a large, diffuse one and presents a potentially more easily rescuable pharmacoperone target. For larger receptors in which the ligand binding site is located in the (large) amino-terminal region (i.e., the calcium-sensing receptor and gonadotropin receptors), the ligands are generally less hydrophobic than those that bind in the TM region. This view is borne out in pharmaceutical development by the observation of the relatively more commonly available peptidomimetics for receptors with intramembrane ligand binding, compared with those that bind ligand at the amino terminus. It is true that there are some nonpeptide compounds that bind gonadotropin receptors and produce effects (van Straten et al., 2002), but these are not true competitors at the ligand binding site (which occurs at the large amino-terminal extension); in fact, binding occurs in or near the membrane-associated components of the receptor (Jäschke et al., 2006).

To function, pharmacoperones need to be small and hydrophobic, so that they can enter cells. Accordingly, the binding site for them is also likely to be small and hydrophobic. These characteristics are also expected to apply for small hydrophobic peptides that bind in the TM region. Accordingly, it is conceivable that these criteria (small hydrophobic ligands that bind to the TM regions of the smaller GPCRs) might circumscribe systems in which pharmacoperones might be expected to work best.

C. Relation between the Overall Receptor Structure and the Structure of the Ligand Binding Site

It is also worth remarking on the observation that chemical structures, which bind to the active site (or at least compete with the ability of agonists to do so), also serve as pharmacoperones, causing misfolded proteins to fold in a way that makes them fold correctly and escape the QCS (Morello et al., 2000; Janovick et al., 2003a; Noorwez et al., 2004; Hawtin, 2006). This observation suggests that there may be a relation between proper construction of the active site and proper overall construction of the receptor. When we (Janovick et al., 2003a) and others (Morello et al., 2000; Bernier et al., 2006; Hawtin, 2006) used pharmacoperones from multiple different chemical classes, we found that if a particular mutant *could be rescued with one class, it could be rescued with pharmacoperones from other chemically unrelated classes*. Mutants that rescued poorly with one

class, rescued poorly with all (Janovick et al., 2003a). Accordingly, even very different peptidomimetic antagonist structures (indoles, quinolones, and erythromycin-derived macrolides), which presumably bind nonidentically to the mutants did something at (or near) the ligand binding site that resulted in rescue by “pulling” the entire molecule into a structure that passed the criteria of the QCS (Janovick et al., 2003a). Similar results were found for distinct mutant, traffic-defective V2Rs (see section III.D.), which may suggest that the active site has been defined at least in part by the overall shape of the molecule that is recognized by the chaperone system and that controls exposure of particular ER retention motifs that serve as mediators of the QCS, functioning only when the receptor is misfolded (Hermosilla and Schülein, 2001; Hermosilla et al., 2004). This appears to be a two-way relation, as placing a template at the ligand binding site also corrects the overall folding of the protein, at least when viewed from the perspective of the cellular QCS. This observation initially seems to be in contradiction with the consideration that endogenous protein chaperones recognize more general errors in the molecule, such as the exposure of a hydrophobic plate (Ellgaard and Helenius, 2001), rather than specific features, such as a defect in a ligand binding site that is specific to an individual receptor. It may be easier to understand the reason for this relation between the binding site and the overall structure of the receptor protein in light of the advantage of binding a ligand at a site that can cause substantive changes in the overall shape of a receptor. These changes would be required for receptor activation and transduction of ligand binding to effector activation, for example. In fact, it is accepted that activation of GPCRs results from agonist-provoked changes in the conformation of the receptor that drives the equilibrium between the inactive and active state in favor of the latter (Gether and Kobilka, 1998; Karnik et al., 2003). The ability of ligands to change the shape of the receptor [and conversely of receptors to stabilize in distinct active conformations in response to different ligands (Lu et al., 2005)] and thereby transfer the signal to domains involved in G protein activation suggests that allosteric GPCR modulators (May et al., 2007) may be potential candidates for pharmacological chaperoning. In this vein, cell membrane-permeant, allosteric modulators may be designed to either aid folding of mutant proteins or misfold overexpressed proteins that may potentially lead to disease, driving the misfolded protein to the degradation pathway.

It is also notable that for both receptors, the distribution of disease-causing mutations is quite broad and includes intra- and extracellular loops, the amino terminus, and the TM regions (Figs. 1 and 3). In the GnRHR, there are (to date) no naturally occurring mutations reported in TM segment 1, extracellular loop (ECL) 3, or intracellular loops 1 or 2. This piece of information alone

could lead to two very different conclusions: either these regions are so important that any mutation leads to lethality (or inability to reproduce) or, alternatively, many are clinically silent and are never reported, even though present. We (Janovick et al., 2002) have constructed a large number of (non-naturally occurring) mutations, including deletions and truncations, in the human and rodent GnRH receptors and found that the vast majority can be rescued by pharmacological means. Even though these mutations interfere with the ability of the receptor to respond, they have not been found in association with disease (Janovick et al., 2002, 2006). This finding suggests that the ability of pharmacoperone rescue is remarkably broad and not limited to “hot spot” areas or particular motifs.

In reconstructing human GnRHR mutants that are associated with disease in orthologous rat or mouse receptor templates, it has been noticed that many of these constructs no longer result in misrouting. This was one of several observations that led to the conclusion that the human GnRHR is delicately balanced between the PM and retention in the ER, whereas rat and mouse GnRHRs are generally routed to the plasma membrane with much higher efficiency (Knollman et al., 2005; Janovick et al., 2006). This trend, which will be discussed in section IV.B., has evolved under substantial selective pressure by several mechanisms (Conn et al., 2006a,b) and costs energy because of the “inefficiency” of needed synthesis of unused protein. Accordingly, this inefficiency may represent a mechanism of post-translational regulation and presents an opportunity for pharmacological intervention.

D. Distribution of Mutations

Fujiwara and Bichet (2005) presented an image of the V2R showing disease-associated mutants (Fig. 1B). There are 188 putative disease-causing mutations in this receptor (sometimes more than 1 at the same site, shown by triangles in the figure). It is clear, by inspection, that the mutation sites are more densely associated with the TM region, an observation that is not surprising in light of the relative conservation of this area and the potential for disturbing the hydrophobic nature of the interaction between the receptor structure and the TM area. The same seems to be true for the GnRHR, although on the basis of far fewer mutations (Figs. 1A and 3). Given the total number of reported mutations, it is remarkable that none have been reported in the amino terminus of the V2R and only 2 have been reported for the GnRHR. It is certainly possible that mutations may occur in this region but go unreported if they are clinically silent. If true, that possibility would minimize the role of this part of the receptor (with the exception of Asn residues involved in glycosylation and intracellular trafficking) in determination of routing. Otherwise all intracellular loops and TM segments of the V2R contain mutations. For the GnRHR, the first

two intracellular loops lack reported mutations. Only two misfolded V2R mutants are located at or near sites recognized as general motifs believed to be involved either in ER export [mutation at R337, involving the E(X)₃LL motif in the carboxyl terminus (Schülein et al., 1998; Krause et al., 2000)] or ER retention [mutation at Glu²⁴², near the two overlapping retinoid X receptor motif at intracellular loop 3 (Hermosilla et al., 2001)] (Figs. 1B and 3; Table 2), whereas in the human GnRHR only one mutation (Arg²⁶²Gln) is located at a potential retinoid X receptor ER retention motif (Gassmann et al., 2005) (Figs. 1A and 3). In both receptors, mutations in the highly conserved motifs E/DRY (located at the boundary of the TM helix 3 and the second intracellular loop and permuted to DRS in the GnRHR and to DRH in the V2R) and D/NPxxY (in the seventh TM helix) have been reported. These motifs have important structural and functional roles in many GPCRs (Gether and Kobilka, 1998; Rovati et al., 2007); GnRHRs and V2Rs bearing mutations in this motif are misfolded receptors that may be partially or completely rescuable by pharmacoperones (Leaños-Miranda et al., 2002; Bernier et al., 2004c,2006; Topaloglu et al., 2006). It has been shown recently that in some GPCRs (including the V2R), mutations in the E/DRY motif promote constitutive receptor endocytosis as a result of increased receptor phosphorylation and arrestin association (Shi et al., 1998; Barak et al., 2001; Wilbanks et al., 2002). Although pharmacoperone rescue of the V2R Arg¹³⁷His mutant showing constitutive internalization may be attributed to inhibition of the constitutive interaction of the mutant receptor with arrestin, Bernier et al. (2004c) show that the effect of the pharmacoperone on cell surface expression and signaling efficacy of the V2R mutant was, rather, attributable to the pharmacological action of the chaperone on a portion of the receptor population that remained intracellularly trapped because of an inability to attain a conformation compatible with ER export. These findings concurrently suggest that the conserved DRY motif is also involved in proper folding and/or ER export of the receptor to the cell surface membrane. Nevertheless, the possibility that pharmacoperones may counteract the effects of mutations, leading to constitutive desensitization via stabilization of the receptor at the cell surface membrane, represents an interesting therapeutic alternative to rescue function of constitutively internalized receptors. In an earlier study by Bernier et al. (2004a), only 7 of the 38 mutants could not be rescued by pharmacoperones.

Because both the GnRHR and the V2R are small GPCRs in comparison to the rest of their superfamily, it is pragmatically easier to prepare mutants for study (i.e., by site-directed mutagenesis). This has added to the facility of use of these mutants for research models. In the case of the GnRHR, all but three [Ser¹⁶⁸Arg, Ser²¹⁷Arg, and the truncated L³¹⁴X (stop)] of the 17 mutants tested were completely or partially rescued

TABLE 2

Location of naturally occurring, loss-of-function GPCRs mutations that compromise or involve believed ER export motifs [YS amino-terminal motif; E(X)₃LL carboxyl-terminal motif], the Arg-X-Arg retention motif, the NxS/T consensus for N-glycosylation (Tan et al., 2004; Dong et al., 2007), the AFNGT motif (present in the gonadotropin receptors that comprise the NxT consensus for glycosylation) (Huhtaniemi and Themmen, 2005), the BBxxB or BBxB motifs [involved in G protein activation (Okamoto and Nishimoto, 1992)], the E/DRYxxI/V and N/DPxxY highly conserved motifs [involved in receptor activation and G protein coupling/recognition (Gether and Kobilka, 1998; Rovati et al., 2007)], and the predicted disulfide bridge between the ECL1 and ECL2. Mutations at sites in bold letters resulted in reported traffic-defective or plasma membrane expression-deficient mutant receptors

| GPCR | YS Motif | NxS/T Consensus | AFNGT Sequence | ECL1-ECL2 Disulfide Bridge Formation | E(x) ₃ LL Motif | RxR Motif | E/DRYxxI/V Motif | N/DpxxY Motif | BBxxB, BBxB Motifs |
|---------------------------------|-----------------|-----------------|--|--|----------------------------|---|--------------------------------------|---|--------------------|
| Rhodopsin | | N ¹⁵ | | C ¹¹⁰ C ¹⁸⁷ G ¹⁸⁸ S ¹⁸⁶ | | | R ¹³⁵ | | |
| V2R | | | | C ¹¹² R ¹¹³ | R ³³⁷ | E ²⁴² | R ¹³⁷ | N ³²¹ (not tested) P ³²² W ³²³ P ³²⁰ | |
| GnRHR LHR FSHR | | | F ¹⁹⁴ A ¹⁸⁹ N ¹⁹¹ | C ²⁰⁰ | | R ²⁶² (in ICL3) | R ¹³⁹ | | R573C |
| TSHR E-BR MC1R | | | | | | R ¹⁶⁰ (in TM4) R ¹⁶² (in TM4) R ¹⁶³ (in TM4) | R ¹⁴² | P ³⁸³ D ²⁹⁴ | |
| MC2R (ACTHR) MC3R MC4R | Y ³⁵ | | | | | | R ¹²⁸ (not tested) | P ²⁷³ (not tested) | |
| CCR5 | | | | C ¹⁷⁸ , C ¹⁰¹ | | R ²²³ (ICL3) | I ¹⁸³ T ¹⁵⁰ | P ²⁹⁹ I ³⁰¹ Y ²⁹⁹ | ΔK228 R319H |

LHR, lutropin (luteinizing hormone) receptor; FSHR, follitropin (follicle-stimulating hormone) receptor; TSHR, thyrotropin receptor; E-BR, endothelin-B receptor; MC1R, melanocortin-1 receptor; MC2R, melanocortin-2 receptor [or adrenocorticotropin receptor (ACTHR)]; MC3R, melanocortin-3 receptor; MC4R, melanocortin-4 receptor; CCR5, chemokine receptor-5.

with pharmacoperones (Conn et al., 2002; Leañós-Miranda et al., 2002; Janovick et al., 2003a; Topaloglu et al., 2006). It has been possible to show that the Ser¹⁶⁸Arg and Ser²¹⁷Arg GnRHRs are mutants in which the thermodynamic changes leading to receptor distortion are too great to effect rescue (Janovick et al., 2006). Accordingly, even though these two mutants are not rescued by pharmacoperones, their failure to route correctly is attributable to misfolding not to an intrinsic inability to potentially participate in particular receptor functions such as receptor activation or G-protein coupling. In the case of misfolded V2Rs, it has been shown that distinct hydrophobic, cell membrane-permeable antagonists (Serradeil-Le Gal et al., 1996; Albright et al., 1998) effectively rescue function of several misfolded, trafficking-defective V2R mutants that cause diabetes insipidus in humans (Morello et al., 2000; Bernier et al., 2004a,b,c). The fact that the effect of these antagonists on mutant V2R expression could not be mimicked by a V2R impermeant antagonist and that the antagonist pharmacoperones did not rescue function of mutants that are normally expressed at the cell surface membrane indicated that the pharmacoperones acted intracellularly to promote maturation and targeting of misfolded mutants to the PM (Morello et al., 2000).

Although not a GPCR, another rescuable molecule is the cystic fibrosis TM conductance regulator (the ion channel that is defective in cystic fibrosis). Unlike the two GPCRs under discussion, in which many different mutations can lead to disease, the same cystic fibrosis TM conductance regulator mutation that results in this

disease is common (approximately 80% of the time)—a deletion of the amino acid at position 508 (Lim and Zeitlin, 2001; Kerem, 2005). This observation initially suggested that the diversity of mutations in the GnRHR and V2R might complicate the search for rescue strategies of intracellularly retained mutant receptors (Oksche and Rosenthal, 1998). Nevertheless, the observations that a number of distinct GnRHR and V2R mutants could be rescued by the same pharmacoperones has clearly challenged this notion (Morello et al., 2000; Janovick et al., 2003a).

IV. Ligand and Receptor Frequency Modulation in Signaling Systems: Implications for Model Selection and the Timing of Pharmacoperone Administration in Vivo

A. Frequency Modulation among Primate Gonadotropin-Releasing Hormone Receptors

Of the V2R and GnRHR, only the latter seems to decode signals that are pulsatile in nature. Nonhuman primates, notably the rhesus macaque, are excellent models for human reproduction that have led to new drugs, medical procedures, and devices. One of the reasons is that the GnRHR of the rhesus macaque, like that of other primates, is sensitive to a complex signal, with both amplitude- and frequency-modulated components from the releasing hormone (Knobil, 1974). This is probably one of the mechanisms by which the cell is able to respond to one ligand with multiple different endpoints,

all having different time constants (Crowley et al., 1985).

There is value in determining the degree of similarity of function among the GnRHRs, as this moiety is the key analog to digital transducer of neural signals that regulates the reproductive endocrine system, and the development of therapeutic approaches will require reliable animal models. Other receptors also may have the ability to transduce a complex signal that is both amplitude- and frequency-modulated and, in the case of the GnRHR, alterations in pulse frequency may provide a means of therapeutic intervention. Because an increased level of FSH over several days seems to be needed to recruit a dominant follicle for ovulation (Wildt et al., 1981), accurate sensing of pulses is important for ovulation. In patients with HH bearing GnRHR mutations, a variable profile of spontaneous pulsatile LH release, from completely apulsatile to decreased frequency and amplitude of pulsatile release, has been found, depending on the particular receptor mutations presented (de Roux et al., 1997, 1999; Beranova et al., 2001; Layman et al., 2001; Meysing et al., 2004). Thus, growing and maturation of ovarian follicles and ovulation cannot occur. The fact that some patients with HH do respond to exogenous (and endogenous) agonists (de Roux 1999; Layman et al., 2001; Meysing et al., 2004) indicates that some degree of GnRHR cell surface membrane expression is present and that the population of mutant receptors so expressed is functional.

The GnRHR itself cycles in rodents and primates (Filicori et al., 1986), and there is the possibility that this process could be neatly controlled by endogenous chaperones (Brothers et al., 2006). To set the stage for in vivo studies, we have examined the molecular aspect of the WT primate GnRHR that might cause this molecule to be amenable to regulation by pharmacoperones.

B. Amino Acids Associated with Control of Plasma Membrane Expression

As described in section III.A., a particular feature of primate GnRHRs is the presence of a Lys residue at position 191, which is located in the second ECL. The presence of Lys¹⁹¹ destabilizes a Cys¹⁴-Cys²⁰⁰ bridge (shown in Fig. 1) that is a critical determinant for primate GnRHRs to pass the QCS of the cell (Conn et al., 2006a,b; Ulloa-Aguirre et al., 2006). Failure of this bridge to form in primates results in production of misfolded mutants that can be rescued with a pharmacoperone or by deletion of Lys¹⁹¹ (Janovick et al., 2006). Because the impact of the Lys¹⁹¹ is largely steric, rat or mouse GnRHRs (which lack a homologous amino acid at this position) seem to form the needed Cys¹⁴-Cys¹⁹⁹ bridge easily and route with high efficiency to the PM (Janovick et al., 2006; Ulloa-Aguirre et al., 2006). In fact, in vitro exposure of rodent GnRHR to pharmacoperones fails to further increase the amount of receptor expressed at the cell surface membrane, whereas in the

human GnRHR pharmacoperone treatment results in a significant increase in the net amount of receptors expressed at the PM (Janovick et al., 2003a,b). For this reason, "inefficient PME" (Conn et al., 2006b) occurs for the primate GnRHR and is apparently a regulatory mechanism that is used by other proteins as well (Petäjä-Repo et al., 2002; Uberti et al., 2005; Petrovska et al., 2005; Vandenberghe et al., 2005).

When human and rat GnRHRs, which differ by 39 amino acids (and the presence of Lys¹⁹¹), are compared, a motif of four nonadjacent amino acids (amino acids 112, 208, 300, and 302) that accounts for the decreased expression of human GnRHR and that participates in regulation of maximal agonist binding (B_{\max}) when Lys¹⁹¹ is present may be identified in humans. Mutational analysis of the primate GnRHR in light of this motif (Janovick et al., 2006; Ulloa-Aguirre et al., 2006) has additionally revealed that Ser²¹⁷ is another critical amino acid closely involved in the control of primate GnRHR cell surface membrane expression. This is recognized as a significant site because Ser²¹⁷Arg is one of only two naturally occurring mutants isolated from patients with disease that cannot be rescued with different pharmacoperones (Janovick et al., 2002, 2003a); it has recently become apparent that this thermodynamically unfavorable substitution results in a mutant in which the Cys¹⁴-Cys²⁰⁰ bridge can never form and is retained in the ER (Janovick et al., 2006). In the human GnRHR, Ser²¹⁷ is associated with formalization of a turn in the peptide backbone that allows alignment of Cys¹⁴ and Cys²⁰⁰. Serine, with a slightly polar nature, small size, and propensity of the side-chain hydroxyl oxygen to hydrogen-bond with the protein backbone, frequently causes it to be found in association with the tight turns of the protein structure. The torsion, resulting from perturbation by a charged amino acid (e.g., Arg) in a membrane bilayer, is apparently too great to be corrected by pharmacoperones. Accordingly, the human sequence has progressively rigidified position 217 to more rigorously control expression at the PM, probably by regulating the probability of formation of the Cys¹⁴-Cys²⁰⁰ bridge (Janovick et al., 2006; Ulloa-Aguirre et al., 2006).

C. Amino Acid Positions Associated with Control of Ligand Binding Affinity

In addition to the previously described GnRHR residues important for ligand binding (see section III.A.), we also found (Janovick et al., 2007b) two amino acid substitutions at positions 54 and 300 (in the rhesus macaque and human, respectively) that affect the affinity of ligand binding without a substantial effect on the B_{\max} of the protein expressed. Because of the position of the amino acid 300 in the receptor molecule, it is easier to imagine that a change at this position (ECL3) might alter ligand affinity by direct interaction with the ligand, although this appears close to a binding pocket (Cui et al., 2000). This case is harder to make with

position 54 located near the cytoplasmic side of the first TM helix (Fig. 1A), although other residues buried in the lipid bilayer appear accessible to ligands (Uberti et al., 2005) and biologically active GnRH analogs are hydrophobic. A change in affinity from a modification at this site may also be a tribute to the interactive nature of the seven TM structures. When the WT rhesus GnRHR is modified to Ser⁵⁴Ala, this change increased affinity of the altered rhesus receptor to the same ligand binding affinity as the closely related WT bonnet and human GnRHRs, thus compensating for the only two other variances in structure; bonnet has Leu⁶ (rather than Ser⁶) and Lys²⁴⁸ (instead of Glu²⁴⁸). Accordingly, the WT human and WT rhesus GnRHRs have markedly decreased affinity for the ligand compared with those of the mouse or rat. The WT bonnet GnRHR is similar in ligand binding affinity to that of the human, but the rhesus macaque has the poorest affinity of all primates. Lys¹⁹¹ found in primate GnRHRs also decreases binding affinity although this is accompanied by a decrease in B_{\max} , an effect previously reported for a chimera of this sequence with the carboxyl tail of the catfish (Maya-Núñez et al., 2000). The fact that nature has relied on changes at two different residues (in different primates) to achieve a decrease in affinity is a tribute to the selective pressure necessary for this to occur.

D. Ligand Binding Affinity as a Squelch Control

The probable selective advantage of this decreased affinity is clearer when one considers that the GnRHR in primates is governed by ligand *frequency* modulation (Fig. 2), rather than by ligand *amplitude* modulation, thus making the distinction of individual pulses more important. The dilution of any square wave, even as small as the pituitary portal system, into a liquid will result in blunting into a sinusoidal wave pattern with alternating low, high, and intermediate levels of GnRH, rather than the “completely on” or “completely off” pattern characteristic of a square wave. In principle, a “completely off” phase may never occur at times of high pulsatility. Accordingly, decreasing the affinity of binding is an effective strategy for ignoring the low level stimuli and producing a crisper response, effectively squelching noise in the system. The evolution toward a system that is more dependent on a frequency-modulated rather than an amplitude-modulated system would benefit by the ability to ignore background noise.

It seems that we are dealing with a primate receptor whose expression is delicately balanced between trafficking to the PM for utilization and retention in the ER; because only a fraction (~50% in this cell type) of the newly synthesized receptor is actually expressed at the cell surface membrane and the rest is presumably degraded without being used, retention in the ER may seem, at first sight, as wasteful. Furthermore, this bal-

ance creates a receptor that is far more sensitive to point mutations compared with that in other animals (Knollman et al., 2005). Despite these apparent costs, there is a strong and convergent evolutionary pressure to restrict the PME of the primate GnRHR consonant with the increased regulatory control of reproduction that occurs in going from nonmammalian vertebrates (birds, fish, and reptiles, animals that produce large numbers of eggs/offspring with a low metabolic investment in each and low survival rates) to mammals, including primates [animals with higher metabolic investment and higher survival rates (Janovick et al., 2006)]. This restriction of PME in humans presents an interesting therapeutic target as pharmacoperones can override this restriction, increasing the percentage of the total GnRHR that is transferred to the PM and decreasing the amount that is retained in the ER (Brothers et al., 2004; Ulloa-Aguirre et al., 2004a). The ability of pharmacoperones to override this process is potentially useful and could result in temporary use to increase ovulation, for example. The fact that a fraction of misfolded, trafficking-defective GnRHRs may be able to reach the cell surface membrane and respond to an agonist also points to pharmacoperones as potential tools for increasing PME of mutant receptors and the effectiveness of the GnRHR system to respond to the endogenous stimulus.

E. Receptor Concentration at the Plasma Membrane as a Gain Control

The sensitivity of the human GnRHR to balance between the PM and ER is seen in several ways. The majority of the mutations causing HH, 14 of the 21 reported mutants, involve change of charge of single amino acids. The remainder affect shape, as four involve the gain or loss of a Cys, one involves Pro, and two are large truncations or deletions (Ulloa-Aguirre et al., 2003, 2004b). Finally, even simple changes in hydrophobicity seem to be associated with compensating changes. Among primates, for example, conversion of Phe¹¹² (present in rhesus, bonnet, and rat GnRHRs) to Leu¹¹² (in human and chimpanzee) is accompanied by a reverse conversion of Leu^{224/225} (rhesus, bonnet, and rat) to Phe^{224/225}. Likewise, the conversion in the human to Leu³⁰⁰ (chimpanzee, rhesus, bonnet, and rat are Val^{299/300}) is accompanied by a complementary conversion at position 155 (Val¹⁵⁵ in human and Leu¹⁵⁵ in chimpanzee, bonnet, rhesus, and rat) (Janovick et al., 2006). These results and the observation that pituitary signaling seems to depend on the pattern of exposure to ligand (Belchetz et al., 1978; Knobil et al., 1980) present the concern that moving this therapeutic approach in vivo, would require some attention to the timing of the administration of the pharmacoperone.

V. Do Pharmacoperones Need to Be Present at the Time of Mutant Synthesis?

As noted, the GnRHR system is sensitive to conveyance of information by both amplitude and frequency-modulated signaling (Knobil et al., 1980). There is a good chance, in fact, that one *raison d'être* for the hypothalamic-pituitary portal system may be related to keeping the GnRH signal in a small volume, so as not to dampen the frequency-modulated component.

It is known that pharmacoperones function by causing misfolded molecules to fold correctly and pass the cellular QCS (Bernier et al., 2004a,b). Figure 4 shows a probable scheme by which GPCRs are assessed by the cell's QCS and can be rescued by pharmacoperones. Because the presently available pharmacoperones for GnRHR and V2R rescue are typically peptidomimetic antagonists (Serradeil-Le Gal et al., 1996; Albright et al., 1998; Janovick et al., 2003a; Bernier et al., 2004c), these must ultimately be removed so that the rescued molecule, stabilized by insertion into the PM, can be occupied and activated by its ligand. Accommodating the need to expose receptor mutants to pharmacoperone *in vivo*—and then remove it to allow occupancy by the endogenous agonist—will probably necessitate pulsatile administration of the pharmacoperone. To determine whether already synthesized (and retained) mutants could be rescued (i.e., in contrast to a requirement that the pharmacoperone be present at the time of synthesis),

protein synthesis or intracellular transport was blocked with cycloheximide or monensin to determine whether pharmacoperones also needed to be present at the time of synthesis to function or whether a previously misfolded/misrouted protein molecule retained by the QCS could be stabilized in a “correct” conformation by *postsynthetic* exposure to a pharmacoperone (Janovick et al., 2007a). Biochemical and morphological studies were performed with both stably and transiently transfected cells using 12 mutants and 10 pharmacoperones selected from different chemical classes. The data (Janovick et al., 2007a) showed that *previously* synthesized mutant proteins, retained by the QCS, are still rescued by pharmacoperones. Similar results were obtained with an ER-retained murine V2R whose cell surface expression was restored by exposure to pharmacoperones even when protein synthesis was abolished (Wüller et al., 2004). These observations suggest that whether the target protein is being synthesized at the time of drug administration need not be considered in determining the pattern of pharmacoperone administration *in vivo*.

In some cases, such as cataracts (lens crystallin aggregation) and neurodegenerative diseases (amyloid aggregation), we suspect that the aggregates may prove to be very stable thermodynamically and rescue of extant aggregates may not be possible by this means. In fact, in an experimental model of cerebral amyloidosis, the main

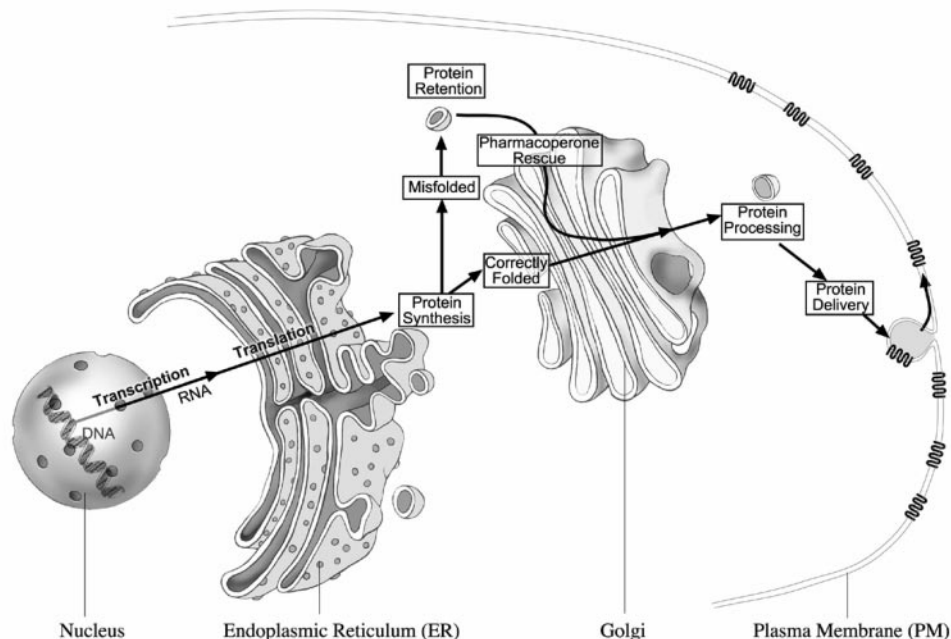


FIG. 4. Cellular sites associated with protein synthesis. Proteins are synthesized in the ER and assessed for overall quality. Folding is facilitated by interaction of the nascent polypeptide with molecular chaperones. Misfolded and misassembled products are retained in the ER and exposed to resident chaperones to attempt folding. Eventually, misfolded proteins are dislocated into the cytoplasm for proteosomal degradation after dissociation of the molecular chaperones. Alternatively, defective proteins may be exported to and retained by the Golgi apparatus, retrotranslocated to the ER where correct folding is again attempted, or diverted to lysosomes for degradation. Mature products are then exported to their final destination (the PM). Pharmacoperones can frequently rescue misfolded proteins by correcting folding and allowing them to escape retention by the QCS and route to the plasma membrane where they are able to bind ligand and couple to the effector system.

effect of pharmacoperones was on prevention of fibril formation by action on fibril intermediates (Soto et al., 1998; Sigurdsson et al., 2000). This drug development approach will eventually result in the development of “lifestyle” drugs that will be taken regularly to prevent the onset of the disease. A second consideration is that the half-life of ER-retained mutants may be short (Robben et al., 2005), which may mean that pharmacoperones need to be present for as protracted a period as possible, if optimal rescue is the goal. Nonetheless, in the case of certain GPCR mutants that are retained in the ER (Illing et al., 2002; Saliba et al., 2002), this seems to be possible and certainly will facilitate therapeutic development.

VI. The Dominant-Negative Effect and Receptor Rescue

Receptor dimerization or oligomerization and interactions with accessory proteins have been well documented and have been proposed to be important determinants of GPCR activity (Conn et al., 1982; Rios et al., 2001; Angers et al., 2002). It seems that GPCRs approach the issue of oligomerization differently, just as some receptors are phosphorylated (whereas others are not) and some receptors bind ligand in the amino terminus (whereas others bind ligands in the lateral plane of the membrane) (Ulloa-Aguirre and Conn, 1998). One reason for this difference might be related to the opportunity for receptor cross-talk affected by heterologous interactions of receptors (Patel et al., 2002). Some receptors are monomeric in the membrane and oligomerize upon ligand binding, whereas others oligomerize as they are synthesized in the ER, an apparent requisite for correct targeting to the cell surface (Rios et al., 2001; Angers et al., 2002). Intracellular association of GPCRs as homo- or heterodimers could lead, in principle, to either cell surface targeting (a dominant-positive effect) (Jones et al., 1998; Kaupmann et al., 1998; White et al., 1998) or to intracellular retention of the complex (DN effect) (Benkirane et al., 1997; Zhu and Wess, 1998; Le Gouill et al., 1999; Brothers et al., 2004; Ulloa-Aguirre et al., 2004a). Furthermore, mutants of several GPCRs may interfere with the cell surface expression of their corresponding WT counterparts through their association in the ER and misrouting of the resulting complex (Zhu and Wess, 1998; Lee et al., 2000; Brothers et al., 2004; Gehret et al., 2006).

Although the GnRH receptor was one of the very first GPCRs shown to oligomerize at the PM as part of normal receptor function (Conn et al., 1982; Cornea et al., 2001), oligomerization in the ER-Golgi complex at the time of nascent protein synthesis and routing to the cell surface is a relatively new concept for GPCRs and suggests that there may also be a function for oligomerization at that early stage (Lee et al., 2000; Lopez-Gimenez et al., 2007; Pin et al., 2007). Perhaps oligomerization of two or more receptors acts to hide exposed hydrophobic

surfaces or ER retention motifs that would otherwise signal an improperly folded receptor and be recognized as such by the quality control apparatus of the cell. Although the exact purpose of oligomerization at the ER or Golgi is currently unknown, one theory is that receptor chaperoning plays a regulatory role in post-translational control of cell surface expression of these and possibly other proteins (Lopez-Gimenez et al., 2007; Milligan, 2007; Pin et al., 2007). In the case of the GABA_B receptor, α_{1D} -adrenergic receptor, β_2 -adrenergic receptor, and FSH receptor, for example, obligatory homodimerization or heterodimerization with related receptors seems crucial for proper folding, maturation, trafficking, surface expression, and cross-talk (Balasubramanian et al., 2004; Salahpour et al., 2004; Uberti et al., 2005; Pin et al., 2007; Thomas et al., 2007).

Of the 21 mutations of the GnRHR reported in patients with HH, 7 mutant receptors were partially functional when expressed in heterologous cell systems, and the remaining mutant receptors were nonfunctional (Janovick et al., 2002; Leaños-Miranda et al., 2002; Topaloglu et al., 2006). When several of the nonfunctional receptors were coexpressed with the wild-type receptor in heterologous cell systems, it was discovered that these nonfunctional receptors also inhibit wild-type GnRH receptor function, a DN effect (Fig. 5) (Leaños-Miranda et al., 2003, 2005). This result is similar to what has been found for other mutant GPCRs (Le Gouill et al., 1999; Lee et al., 2000; Gehret et al., 2006), including the V2R (Zhu and Wess, 1998). In this receptor, coexpression of laboratory-manufactured V2R truncated mutants with the WT V2R inhibited cell surface membrane expression of the wild-type receptor by forming intracellular heterodimeric complexes (Zhu and Wess, 1998). Creation of a protein chimera in which the green fluorescent protein sequence was added to the carboxyl terminus of the wild-type GnRHR sequence allowed the use of confocal microscopy to localize wild-type receptors that were coexpressed with the DN mutant receptors. The DN action that the mutant GnRH receptors have on the wild-type receptor seems to be due to ER retention of an aggregate of wild-type and mutant proteins (Brothers et al., 2004; Ulloa-Aguirre et al., 2004a). The wild-type and mutant receptors seem to form oligomers in the ER, and those oligomers were retained and presumably degraded. We are unaware of similar reports for naturally occurring mutants of the V2R, with the exception of a nonfunctioning splice variant identified in the rat kidney that exhibited marked down-regulation on WT V2R surface expression (Sarmiento et al., 2004).

The function of mutant GnRHR pairs associated with compound heterozygous patients showing complete or partial forms of HH has been analyzed (Leaños-Miranda et al., 2005). This study was done to examine potential interactions *between* misfolded mutants that may influence net receptor function and response to pharmacological rescue. Nine pairs of GnRHR mutants and an un-

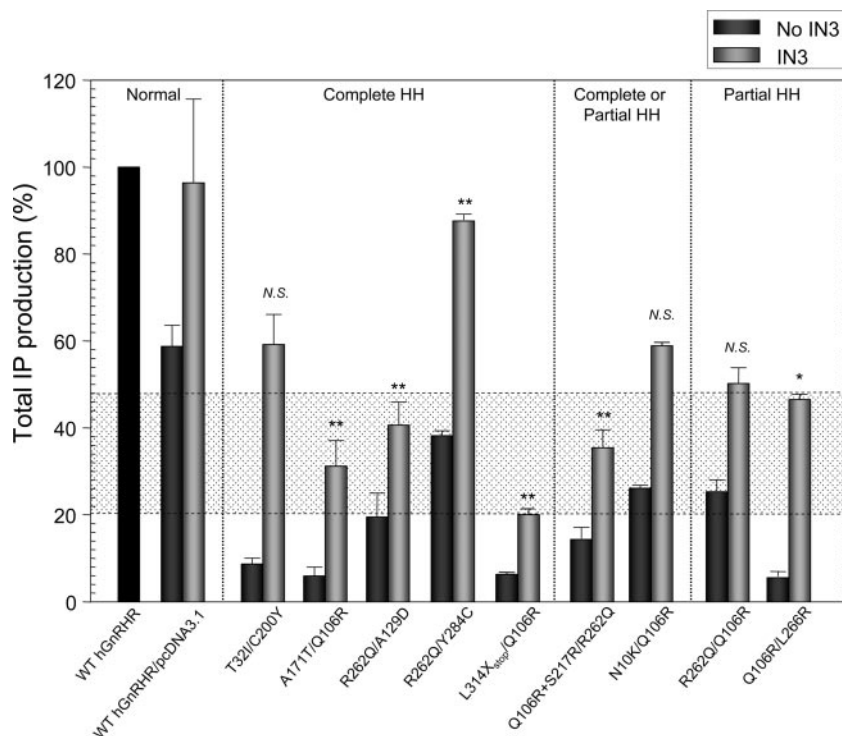


FIG. 5. Maximal agonist-stimulated inositol phosphate production (IP) in COS-7 cells coexpressing different human GnRHR (hGnRHR) mutant combinations separated according to the clinical phenotype reported in patients with HH (Ulloa-Aguirre et al., 2004b). Maximal IP production in cells transfected with the WT human GnRHR cDNA is taken as 100%. Cells were cultured in the absence (black bars) or presence (gray bars) of a pharmacoperone. Data represent the means \pm S.E.M. of at least three independent experiments. The upper discontinuous horizontal line is set at -2 S.D. of the maximal agonist-stimulated IP production from cells cotransfected with the WT human GnRHR and the empty vector and cultured in the absence of pharmacoperone (IN3), whereas the lower horizontal line denotes -2 S.D. of the mean maximal IP production exhibited by the coexpression of the heterozygous hGnRHR mutants leading to complete or partial (Asn¹⁰Lys/Gln¹⁰⁶Arg) or to partial (Arg²⁶²Gln/Gln¹⁰⁶Arg) forms of HH. The shaded area delimits the functional level of hGnRHR mutants that upon pharmacological rescue would predict partial correction of the HH; levels above this area would lead to complete recovery and restoration of a normal phenotype, whereas levels below the area would predict complete or nearly complete failure to pharmacological rescue. Values above gray bars indicate the level of significance. *, $p < 0.05$; **, $p < 0.01$; NS, nonsignificant versus WT human GnRHR plus empty vector in the absence of the pharmacoperone. Modified from Leañes-Miranda et al. (2005) with permission. Copyright 2005, The Endocrine Society.

reported combination [Leu³¹⁴X(stop)/Arg²⁶²Gln] were studied. Coexpression of each pair of mutants in Cos-7 cells resulted in either an active predominant effect (Gln¹⁰⁶Arg/Leu²⁶⁶Arg, Ala¹⁷¹Thr/Gln¹⁰⁶Arg, Thr³²Ile/Cys²⁰⁰Tyr, and Arg²⁶²Gln/Ala¹²⁹Asp mutant GnRHR pairs), an additive effect (Arg²⁶²Gln/Gln¹⁰⁶Arg, Asn¹⁰Lys/Gln¹⁰⁶Arg, and Arg²⁶²Gln/Tyr²⁸⁴Cys mutant GnRHR pairs), or a DN effect [Leu³¹⁴X(stop)/Gln¹⁰⁶Arg, Gln¹⁰⁶Arg + Ser²¹⁷Arg/Arg²⁶²Gln, and Leu³¹⁴X(stop)/Arg²⁶²Gln mutant GnRHRs]. For all combinations, addition of a pharmacoperone increased both agonist binding and effector coupling (Fig. 5). Although effective, the net ability to rescue with a pharmacoperone was unpredictable because responses could be either similar or higher or lower than those exhibited by the less affected mutant. The clinical phenotype in patients expressing complex heterozygous alleles seems to be dictated both by the contribution from each mutant and by a DN effect similar to that reported for mutants and wild-type receptor. These studies suggest that, depending on the genotype, partial or full restoration of receptor function in response to pharmacological chaperones may be achievable goals in patients bearing inactivating mutations in the GnRHR gene. In this scenario,

pharmacoperones may either correct folding of the mutant receptors, allowing the possibility that one or both of the mutants may escape the QCS and traffic to the PM or interfere with aggregation and degradation of the mutant receptors. In this vein, the observations that a synthetic β_2 -adrenergic receptor TM helix 6-derived peptide inhibited dimerization of this receptor (Hebert et al., 1996) and that aggregation of secretory proteins may be inhibited by small, cell-permeant synthetic ligands (Rivera et al., 2000) give further support to the latter possibility.

The pharmacoperone rescue of mutants coexpressed with WT receptor resulted in recovery of the WT as well (Brothers et al., 2006). This observation suggests that in vivo use of such compounds could be highly effective in overriding the DN effect of a mutation on the WT, as well as in rescue of the mutant itself.

VII. Will Pharmacoperone Drugs Be Species-Specific? Selecting the Correct Models for Drug Development

Pharmacoperones reveal that a large percentage of the human, but not rat, GnRHR is retained and never

arrives at the PM. The further observation that pharmacoperones increase the PME of the WT human GnRHR itself, but not the rat counterpart, means that the human protein was only partially transferred to the PM and the remainder was apparently retained in the ER and eventually degraded, as has been found for other intracellularly retained GPCRs (Petájá-Repo et al., 2001; Andersson et al., 2003; Cook et al., 2003; Lu et al., 2003). Moreover, the observation that the human GnRHR is more susceptible to mutations than the rat or mouse ortholog supports the view that the human receptor is very precariously balanced between retention in the ER and routing to the PM and provides an underlying mechanism for a novel level of post-translational regulation of WT proteins (Conn et al., 2006a,b; Janovick et al., 2006). Retention of WT proteins is sometimes referred to as “inefficient” protein utilization and there is evidence that this mechanism is used by a variety of systems (Petájá-Repo et al., 2001; Andersson et al., 2003; Cook et al., 2003; Lu et al., 2003). It is not seen in rat or mouse GnRHRs, which route very efficiently to the PM (Janovick et al., 2003b), suggesting that the expression of wild-type receptors that route heavily to the PM and are not retained by the ER QCS normally may be less sensitive to point mutations, as exemplified by the rat and mice GnRHRs. Even among rodents, recent studies have identified an evolutionary trend in which very modest molecular changes (one single carbon) dramatically alter the character of protein trafficking and result in new regulatory potentials (Knollman et al., 2005).

In the case of the human GnRHR, the creation of an inefficiently expressed GnRHR seems to have evolved under strong pressure and with the use of several distinct strategies. This inefficiency results in considerable susceptibility to mutation and apparent waste of protein but provides a considerable advantage in terms of regulation and function, including responses to differing pulsatile stimuli. These observations, in addition to identifying an interesting level of post-translational regulation, also suggest that this inefficiency, at least for the primate GnRHR, continues to evolve and drugs may have high species specificity. Thus, in designing new drugs for their use as pharmacoperones, it is important to consider the evolutionary aspects that determine the particular structure-function relationship of the target protein. Thereafter, screening for and identification of drugs should not be problematic, because human receptor sequences can be expressed in cell cultures and combined effectively with high throughput screening.

One approach undertaken to determine the impact of GnRHR routing differences between species was the reconstruction of mutations identified from patients with HH in mouse and rat GnRHR sequences. In the human, these changes of single amino acids caused disease because they resulted in misfolding and subsequent identification by the cellular QCS as being defective.

They were retained in the ER for destruction (Knollman et al., 2005). Surprisingly, many of these mutations frequently had very little or no effect in rat or mouse sequences. The fact that pharmacological chaperones could rescue them indicated that the effect of these mutations was to cause protein misfolding and a resultant loss of the ability to move from the site of synthesis to the PM. It was not a loss of the ability to bind ligand or interact with the effector because the rescued proteins could function identically to the wild-type molecules.

How is the rat GnRHR different from the human GnRHR? Considering that there are 39 amino acid differences between the rat and human GnRHR sequences and a seemingly endless number of mutants to explore all the combinations, identification of the important differences between the rat and the human GnRHR was initially approached by locating the thermodynamically unfavorable changes (Janovick et al., 2006), figuring that these might be the most important. Interestingly, there were only three, and these were located in close physical proximity to the Lys¹⁹¹ and to the Cys¹⁴-Cys²⁰⁰ bridge. It was also interesting that these all involved the loss or gain of a Ser or Pro, both of which are associated with *introducing a bend in the protein backbone* and setting the alignment between the second ECL and the amino terminus. The rest of the motif was identified by making guesses about the physical relation between amino acids in the three-dimensional state. With this information, human receptors that were modified to be rat-like at four residues were created. These were expressed at the higher levels associated with rat receptor and lacked the requirement for the Cys¹⁴-Cys²⁰⁰ bridge, another feature of the rat GnRHR (Janovick et al., 2006). The spatial alignment was quite important because the two Cys residues had to be within the size of one water molecule for the bridge to form (Ulloa-Aguirre et al., 2006). When the bridge forms, the human GnRHR is recognized by the cellular QCS as correctly folded. When it does not form, it is viewed as defective and retained (and then presumably destroyed) in the ER. The cell is apparently exploiting this approach as a means of controlling routing in the normal function of healthy cells. Indeed, among GnRHR mutants associated with disease in humans, 12 of 18 are associated with changes in the charge of single amino acids; the rest are insertion or removal of prolines that forcibly bend the receptor structure or the insertion or removal of cysteines that result in loss or gain of Cys bridges (Ulloa-Aguirre et al., 2004b).

Several other factors are also important from the structural point of view. First, the conversion of the Gly²¹⁶ (mouse) to Ser²¹⁶ (rat) that altered the DN effect in these species (Knollman et al., 2005) is a mutation that results in torsion of the second ECL and is, accordingly, in a position that reasonably might affect the relation between the Cys¹⁴ and Cys²⁰⁰, attaching the amino terminus to the second ECL (Ulloa-Aguirre et al.,

2006). Second, mutants Ser¹⁶⁸Arg and Ser²¹⁷Arg are in a previously reported unrescuable zone (Janovick et al., 2003b) characterized by a complete lack of response to several different classes of pharmacoperones that otherwise successfully rescue other mutants *in vitro*; this is a quite rare circumstance, as the vast majority of mutants are rescuable by all classes (Ulloa-Aguirre et al., 2004a). Although these sites might be recalcitrant to rescue because they may be important for ligand-receptor interactions or receptor activation, it is now evident that there is a different explanation: the physical relation between TM helices 4 and 5 to the positioning of ECL2 makes it attractive to consider that (charge altering) mutations in these two residues exert their influence by regulating the position of this loop and the intimacy of Cys¹⁴ and Cys²⁰⁰. Because of charge considerations, the thermodynamically unfavorable exchange replacing Ser by Arg (in the lateral plane of the membrane) probably moves this loop into a position from which the formation of a Cys bridge is improbable and the mutant never passes the cellular QCS even in the presence of pharmacoperones.

Because the rat lacks the “extra” amino acid at position 191, the homologous position of amino acid 217 in the human is 216 in the rat, the very same position that distinguished the rat from the mouse by interconversion of a Gly (mouse) to Ser (rat). The amino acids selected are interesting, because serine, with a slightly polar nature, small size, and propensity of the side-chain hydroxyl oxygen to H bond with the protein backbone, also causes it to be found in association with tight turns of the protein structure. Gly, on the other hand is very flexible.

In considering the molecule as a whole, the rat to human modifications associated with orientation of ECL2 (positions 7, 168, 189, and 202/203) *all* involve the gain or loss of Pro or Ser. Pro forms a five-membered nitrogen-containing ring, a feature that causes it to be found in very tight turns in protein structures (i.e., where the polypeptide chain must change direction). It is thus evident that the GnRHR peptide backbone is being bent to control the relation between the Cys¹⁴ and Cys²⁰⁰ and controlling the probability of formation of the bridge. This means that any drug capable of promoting or stabilizing formation of this bridge, critical for cell surface membrane expression of the human GnRHR, may be potentially useful as pharmacoperone.

Toleration, let alone strong and convergent evolutionary pressure for such mutational liability, along with the burden of inefficient function, suggests that this post-translational regulation is extremely important in advanced mammals. It provides a mechanism by which proteins, through interactions with the QCS, can rapidly respond to demand, even without protein synthesis. The latter may imply the existence of a “reserve pool” of receptors that can be recruited during high-demand conditions; in this vein, the observation that pharmacoper-

one-provoked GnRHR rescue occurs even after previous exposure to inhibitors of protein synthesis (Janovick et al., 2007b) supports this view. This mechanism may be tightly coupled to increased synthesis of the receptor and the subsequent escape from the proteasomal degradation resulting from saturation of the QCS. Both possibilities remain merely speculative at this time, and it is clear that further efforts to identify specific interactions between receptors and endogenous chaperones and/or escort-like proteins that divert newly synthesized receptors from degradative pathways may elucidate conditions that allow a fraction of receptors to escape the QCS and be targeted to the cell surface membrane to interact with agonist.

The observation that only a fraction of primate WT GnRHRs are able to reach the cell surface and interact with the agonist also rises the question of why Nature allows wasting of ~50% of newly synthesized receptor in primates but not in rodents. Why construct a receptor that is so delicately balanced between the PM and the ER that a single charge change results in disease? Besides providing a pool of receptors that can be called upon to be available in times of need, this type of post-translational regulatory mechanism may serve to allow the primate pituitary gland (a more precise modulation of gonadotropin release) and, hence, maturation of a single oocyte during each ovulatory cycle. In this scenario, the complexity of reproduction seems to have evolved as the investment in creating a single offspring increased. A close look at the GnRHR in animals whose sequences seemed odd has revealed that several species display marked differences in their reproductive patterns compared with their evolutionarily close relatives. Among rodents, animals with large litters, only the guinea pig is known to have an added amino acid (Glu) at position 191 of the GnRHR. Interestingly, the guinea pig, a hystricomorph that diverged very early in rodent evolution, has a long luteal phase (a primate characteristic). Most nonrodent mammals such as cows, sheep, pigs, dogs, and horses also contain Glu¹⁹¹, suggesting that the loss of an amino acid in the homologous position is a specialization associated with very short reproductive cycles in rats and mice. Unlike all other reported mammalian sequences, the opossum (a nonplacental mammal that places fetuses in a marsupium and “births” by a mechanism that differs from other mammals) has an uncharged, racemic Gly¹⁹¹ that may reflect the early divergence of this group and specializations needed for this relatively unique form of reproduction compared with that of other mammals.

Is this a unique exercise of the human GnRHR? Although presently there are no other GPCRs for which this evolved restriction of PME has been documented at the amino acid level, it is possible that this event may be more generally applicable in light of recent reports of other WT receptors that are also inefficiently expressed (Petäjä-Repo et al., 2000; Couzin, 2002; Saito et al.,

2004; Uberti et al., 2005; Petrovska et al., 2005; Pietilä et al., 2005; Vandenberghe et al., 2005). It will be necessary to determine whether other proteins follow the same regulatory mechanism. Candidate proteins for this delicate balance between the ER and PM are likely to show sensitivity to single point mutations (naturally occurring or created) in which net charge is added or altered and such mutations will be distributed generally throughout the molecule, as is the case with the GnRHR (Janovick et al., 2003a; Knollman et al., 2005). Application of these criteria may enable selection of candidate molecules for subsequent study.

VIII. Endogenous Chaperones as a Potential Site for Therapeutic Intervention

Like many other proteins, GPCRs are subject to evaluation by the QCS, and misfolded proteins are retained in the ER. The individual constituents of the QCS are the molecular chaperone proteins of the ER. Because these chaperone proteins can control routing, they may also be bona fide therapeutic targets. Several ER chaperones and GPCR-interacting proteins have been shown to interact with GPCRs, modulating their intracellular retention/trafficking and targeting to the PM (Dong et al., 2007). Some of these chaperones and GPCR-interacting proteins behave as promoters of GPCR folding and/or receptor transport to the cell surface defining the phenotypes of the receptors at the PM [e.g., ATBP50 for the angiotensin-2 receptor; Usp4 for the α_2 -adrenergic receptor; and RAMPs in the case of the calcium-sensing receptor (Bouschet et al., 2005; Wruck et al., 2005; Milojevic et al., 2006)], whereas others [e.g., BiP/Grp78 and Grp94 for the dopamine-1 receptor, glycoprotein hormone, and rhodopsin and DriP78 for the dopamine-1 receptor (Anukanth and Khorana 1994; Bermak et al., 2001; Sifroi-Fernández et al., 2002; Mizrachi and Segaloff, 2004)] participate in their intracellular retention. In the case of the GnRHR and the V2R, only the interaction of these receptors with the chaperone calnexin has been documented (Morello et al., 2001; Brothers et al., 2006).

Calnexin, one component of the QCS of the ER, interacts with sugar molecules (monoglycosylated *N*-linked chains) and the peptide chain of newly synthesized receptor folding intermediates (Helenius et al., 1997; Vassilakos et al., 1998; Schrag et al., 2003). Because a proportion of the human GnRHR is retained in the ER (Janovick et al., 2002; Brothers et al., 2004), we recently examined the possibility that calnexin may mediate ER retention of the WT GnRHR (Brothers et al., 2006). Furthermore, because the rat GnRHR seems to be more efficiently trafficked to the PM than the human GnRHR, we compared the effect of calnexin on these receptors as well. Expression of the WT human GnRHR with calnexin decreased receptor expression by approximately half, diminishing receptor-mediated second messenger production. The

rat receptors were also retained by calnexin, but because a larger proportion of the rat GnRHR normally reaches the PM, there was no effect on maximal receptor signaling. Calnexin seems to retain a proportion of both human and rat GnRHRs in the ER, probably by means of a physical interaction between the proteins. In the presence of a pharmacoperone, there is a calnexin-mediated increase in human GnRHR signaling, probably reflecting an increase in PME. The pharmacoperone-stabilized receptors seemed to be more efficiently routed to the PM. Thus, calnexin seems to act as a quality control protein for the GnRHR by retaining misfolded receptors and steering properly folded receptors to the PM. Calnexin did not affect rat receptor-mediated second messenger production either with or without the pharmacoperone when expressed with similar amounts of cDNA as the human receptor, an interesting observation when one considers that this chaperone mediated a 40% reduction in rat receptor surface expression. Nearly all of the rat GnRHR is properly folded and expressed at the PM (Lin et al., 1998; Arora et al., 1999; Maya-Núñez et al., 2000); such very high expression is consistent with the observations that the rat receptor is not rescued by pharmacoperone exposure. Only when the cDNA of rat receptor was decreased 12.5-fold, did the additional calnexin decrease inositol phosphate production. As for virtually all cells, the Cos-7 cells used in this study express endogenous calnexin (Allen et al., 2001). When siRNA was used to knock down the transfected calnexin, the human GnRHR signaling was restored. Calnexin siRNA had little effect on the already robust rat GnRHR signaling.

The addition of the intracellular carboxyl-terminal extension or deletion of Lys¹⁹¹ from the human GnRHR dramatically increased PME in both cases (Lin et al., 1998; Maya-Núñez et al., 2000; Leños-Miranda et al., 2003). Calnexin coexpression with human GnRHRs chimeras bearing this domain or without Lys¹⁹¹ no longer affected signaling. Thus, either calnexin does not interact with these modified receptor molecules (particularly with the desLys¹⁹¹ form of the receptor), or, more likely, any reduction in membrane expression did not diminish second messenger production, as is seen with the rat GnRHR. The carboxyl-terminal extension is important in cell surface membrane expression of the GnRHR through a dual effect: its presence decreases internalization rates, resulting in increased net membrane expression (Willars et al., 1999; Brothers et al., 2002), and it also functions to increase the stability of the receptor, promoting its transport to the cell surface.

In the case of the V2R, Morello et al. (2001) showed that calnexin interacts with both the WT and misfolded mutant V2Rs [Arg³³⁷X(stop) and Ser³¹⁵Arg mutants]. However, the half-lives of the receptor-calnexin interactions varied, depending on the particular receptor; re-

tention of misfolded V2Rs was associated with longer interaction times between the mutant receptors and calnexin, suggesting that this chaperone could play a role in the intracellular retention of misfolded GPCRs.

The effect of pharmacoperones on WT GnRHR rescue and the observation that misfolded human mutant GnRHR and V2Rs are retained by calnexin suggest that this protein chaperone recognizes precursors of the mature protein as well as misfolded proteins. In addition to showing that a proportion of the WT human GnRHRs are retained by calnexin, our studies also suggest phosphorylation-dependant regulation of GnRHR PME by calnexin as the signaling output of the receptor was either decreased or increased, depending on whether Ser⁵⁰⁴ or Ser⁵⁸³ in calnexin was present. The increased control over the human GnRHR signaling may be advantageous when the complicated human reproductive cycle is regulated but prove disadvantageous when mutations are introduced, as in HH and nephrogenic diabetes insipidus.

It is clear that exogenous manipulation of ER retention mechanisms may be a useful tool to influence receptor trafficking and function. Moreover, several studies have shown that manipulation of components involved in the ER export machinery may also selectively influence receptor PME and function. This is the case of the small GTPase Rab1 protein, a member of the Rab GTPase family of proteins (Martinez and Goud 1998); Rab1 is specifically localized in the ER and Golgi apparatus and regulates anterograde transport from the ER to and through the Golgi of several proteins (Yoo et al., 2002; Wu et al., 2003; Filipeanu et al., 2004, 2006). Attenuation of Rab1 function by expressing DN Rab1 mutants or siRNA-mediated depletion of endogenous Rab1 inhibited cell surface expression of a number of endogenous GPCRs (including the angiotensin-1 receptor and the β_2 -adrenergic receptor) and promoted their accumulation in the ER and the Golgi (Wu et al., 2003; Filipeanu et al., 2004). The overall data are consonant with the idea that control of the level of molecular chaperones and GPCR-interacting proteins may create an underappreciated therapeutic target.

IX. In Vitro and in Vivo Studies with Pharmacoperones: How Close Are We to the Transferring of Discoveries from the Laboratory Bench to the Bedside?

Several approaches have been applied to salvage misfolded proteins in vitro. Among these are the use of physical methods (Denning et al., 1992; Brown et al., 1997; Matsuda et al., 1999; Zhou et al., 1999), nonspecific, low molecular weight protein-stabilizing compounds such as polyols (chemical chaperones) (Sato et al., 1996), genetic modification of mutant proteins (Cheng et al., 1995; Schülein et al., 2001; Maya-Núñez et al., 2002), and use of pharmacoperones. All of these

approaches correct errors in folding and restore activity by correcting routing (Zhou et al., 1999; Morello et al., 2000; Conn et al., 2002; Petäjä-Repo et al., 2002). Certain low molecular weight compounds such as glycerol, trimethylamine *N*-oxide, 4-phenylbutyric acid, and deuterated water can stabilize proteins against thermally induced denaturation (Rubenstein et al., 1997). Although chemical chaperones can rescue some misfolded proteins, they are nonspecific in regard to rescuing a designated protein and might potentially increase secretion of many different proteins in various cellular compartments, leading to inappropriate changes in the levels and/or secretion of many proteins, which may be highly undesirable. In fact, in some situations high concentrations of chemical chaperones might alter folding of some molecules in a manner that they may potentially promote polymerization/aggregation of certain conformationally defective proteins (Yang et al., 1999). It has been observed, however, that some agents such as glycerol, 4-phenylbutyric acid, and trimethylamine *N*-oxide selectively increase the secretion efficiency of α_1 -antitrypsin without influencing the secretion efficiency of other proteins or decreasing proteasomal degradation (Burrows et al., 2000; Perlmutter, 2002). The mechanism whereby the former chaperones may selectively influence secretion is unknown, although up-regulation of the chaperone system has been proposed to explain why misfolded variants of α_1 -antitrypsin, but not those of other wild-type proteins, are secreted more efficiently (Cohen and Kelly, 2003). Chemical chaperones require high concentrations for effective folding of mutant proteins and hence are too toxic and unsuitable for clinical applications. Genetic approaches in which modifications are introduced to an already defective protein have been used to rescue function of conformationally abnormal molecules. These approaches either overexpress or stabilize molecules rendered unstable by genetic defects. Genetic strategies to rescue misfolded proteins do not, in theory, provoke global changes in the ER secretory activity unless a particular agent capable of enhancing the transcription of several genes is used to obtain such an effect (Cheng et al., 1995). Nevertheless, genetic approaches are probably redundant as therapeutic interventions because if it were possible to access the gene sequence, the primary error could be directly addressed. The preceding considerations leave pharmacoperones as the most promising therapeutic approach to treat conformational disorders, including HH and nephrogenic diabetes insipidus caused by conformationally defective GnRHR and V2Rs.

In addition to the misfolded GnRHR and the V2R mutants, for which nonpeptide antagonists have proved to be useful as pharmacoperones, there are other conformationally defective GPCRs in which these drugs have been demonstrated to be efficacious in rescuing function or in preventing abnormal accumulation of the defective molecule in in vitro systems. In retinitis pigmentosa,

folding and rescue of the P23H mutant rhodopsin associated with this incurable retinal degenerative disease has been achieved by providing cells with 11-*cis*-7-ring-retinal, a seven-membered ring variant of 11-*cis*-retinal, the chromophore of rhodopsin that plays a central role in the photoactivation process (Noorwez et al., 2003, 2004). In the case of PME-deficient μ -opioid receptors and melanin-concentrating hormone receptor-1 mutants, different cell-permeable agonists and antagonists have shown to effectively enhance cell surface expression of the mutant receptors (Chaipatikul et al., 2003; Fan et al., 2005). Regarding other conformationally defective proteins, short β -sheet breaker peptides have been designed for blocking the conformational changes and aggregation undergone by β -amyloid (Soto, 2001). These synthetic minichaperones, which have a structure homologous to the central hydrophobic region of the fibril aggregate, inhibit and dissolve β -amyloid aggregates in vitro (Soto et al., 1998). In transthyretin amyloidogenesis, several small molecules may bind with high affinity to the unoccupied binding sites within the transthyretin molecule, leading to stabilization of the native state of the protein and decreasing the concentration of the intermediate species and amyloid formation (Cohen and Kelly, 2003; Hammarström et al., 2003). The competitive α -galactosidase A inhibitor, 1-deoxygalactonojirimycin, increases the activity of the R301Q mutant form of this enzyme (whose retention in the ER leads to the lysosomal storage disease, Fabry's disease, in humans) and facilitates its ER export and transportation to lysosomes in fibroblasts expressing the mutant enzyme (Fan et al., 1999). Similar results have been obtained by treating fibroblasts from patients with Gaucher's disease (which results from mutations in lysosomal β -glucosidase leading to the accumulation of glucosylceramide in macrophages) with the enzyme inhibitor *N*-(*n*-nonyl) deoxyojirimycin (Sawkar et al., 2002).

Some of the in vitro studies described above have been followed by studies in experimental animals. A rat model of cerebral amyloid- β deposition has been developed by injecting solubilized amyloid- β peptide into the amygdala of rats. In this model, administration of β -sheet breaker peptides led to reduction in amyloid- β deposition but mainly prevented fibril formation (Soto et al., 1998; Sigudsson et al., 2000). Similar results have been found in a transgenic mouse model of Alzheimer's disease (Permanne et al., 2002). In transgenic mice expressing the Arg³⁰¹Gln mutant of α -galactosidase A, oral administration of the inhibitor 1-deoxy-galactonojirimycin elevated the enzyme activity in the heart by 4.8- to 18-fold and to a lesser extent in kidney, spleen, and liver (Fan et al., 1999).

There are not a large number of clinical trials on pharmacoperone effectiveness, but one stands out (Bernier et al., 2006). In this study of in vivo human trials, five patients with nephrogenic diabetes insipidus due to V2R mutations (Arg¹³⁷His, Trp¹⁶⁴Ser, and 185-

193del) were assessed. The efficacy, over a short term, of the peptidomimetic V1_AR/V2R antagonist SR49059 (Serradeil-Le Gal et al., 1993; Bernier et al., 2004c) was examined and revealed a drop in urine production and water intake as well as a significant increase in urine osmolarity (Bernier et al., 2006); in parallel in vitro experiments, cell surface expression and function of all three V2R mutants were rescued by exposure of transfected COS-1 cells to the pharmacoperone. Although the chemical development of the drug used has been halted due to potential interference with the cytochrome P450 metabolic pathway, the study represents an important proof of principle. It is our prejudice that optimization of the route and pattern of administration will also improve the efficacy of this approach for this and other indications.

X. Conclusions

As we prepare to go down the "translational highway," moving an observation in basic science to clinical utility, it is important to consider what lessons we may be able to take from the basic science studies that will facilitate the clinical work. In this review we have examined in detail the two best characterized systems for GPCR rescue and draw conclusions about what may constitute the most appropriate systems to study and how the findings from bench work have an impact on the likely direction as in vivo studies are attempted. We anticipate that regulation of the routing of cellular proteins will provide the opportunity for novel drug development for repair of misrouting of protein mutants and for WT proteins that are normally inefficiently routed.

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