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Peptidomimetic Regulation of Growth Hormone Secretion

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I. Introduction

HORMONES are generally released episodically, yet to our knowledge no attempt has been made to design drugs for treating hormone deficiencies that imitate or amplify the endogenous oscillators governing pulsatile hormone release. For example, although GH is normally secreted in a pulsatile manner with peaks occurring approximately every 3 h, GH deficiency is treated by injecting recombinant GH either once daily or every 3 days. Clearly, this dosing regimen does not result in a physiological GH profile and urges the development of a more natural approach for the treatment of GH deficiency. For convenience to the patient, a pathway was sought that could be activated by a drug given orally once daily. By design, conventional peptides were excluded, because the molecular template had to be readily manipulable for optimization of oral absorption and pharmacokinetics. A receptor that controlled GH release by interaction with an endogenous small molecule was unknown, necessitating a heterodox approach to drug discovery. This reverse pharmacology involved: 1) establishing assays for the functional endpoint, stimulation of episodic GH release; 2) understanding at the cellular level how known peptides controlled GH release so that new specific targets of intervention could be selected; 3) identifying selective GH secretagogues of appropriate structure for optimization of pharmacokinetic properties; 4) characterizing and cloning the receptor for these ligands; and 5) identifying the receptor's natural ligand. This review provides a summary of points 1–4, focusing on the discovery and properties of new GH secretagogues. These molecules were subsequently shown to be peptide mimetics of the GH-releasing peptides (GHRPs) first identified by the pioneering work of Bowers, Momany, and colleagues (1–3); for a comprehensive review of GHRPs the reader is referred to Refs. 4–9. The natural ligand for the receptor activated by GHRPs and its peptidomimetics remains elusive, but now that the receptor has been cloned and distributed, we anticipate its ligand will soon be discovered.

II. Identification of Peptidomimetic GH Secretagogues

A. Mechanism of action of GHRH, GHRP-6, and somatostatin

The objective was to somehow amplify the GH-secretory pathway with a small orally active molecule. This required a detailed knowledge of the mechanisms governing GH release at the cellular biochemistry level as well as understanding the interplay between the central nervous system and the anterior pituitary gland. Two known hypothalamic hormones, GHRH and somatostatin, are key regulators of GH release from somatotrophs in the pituitary gland; GHRH stimulates GH secretion, and somatostatin is inhibitory (10–13). In addition to GHRH, a synthetic hexapeptide, His-D-Trp-Ala-Trp-D-Phe-Lys-NH_2 (GHRP-6), first described by Bowers' group, is also a potent GH secretagogue (1, 2). The GHRPs were first described in 1981 (3), before GHRH (14, 15), but until recently their receptor had not been defined (16).

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The obvious approach was to identify nonpeptide mimetics of either GHRH or GHRP-6; however, in contrast to antagonists, the probability of finding nonpeptide agonists of peptides was considered to be very low. The only precedent was morphine and its congeners, which are nonpeptide mimetics of the opioid peptides. Therefore, from a medicinal chemistry perspective, a more reasonable approach to amplify GH release with a small molecule was to design a nonpeptide antagonist of somatostatin. However, the potential for extrapituitary effects of a nonselective somatostatin antagonist made somatostatin a less than ideal target; moreover, before the cloning of somatostatin receptor subtypes, screening for selectivity was not an option.

In spite of the historical difficulties of identifying peptidomimetics, GHRH and GHRP-6 were excellent drug targets. *A priori*, a peptidomimetic of GHRH seemed the most obvious choice because it had been studied extensively. However, structure-activity relationships indicated that the size of the molecule could not be reduced below 29 amino acids without a significant loss in activity (17). The peptide GHRP-6 was of ideal size, but because its receptor had not been identified, and cell lines responsive to GHRP-6 were unknown, high volume screening for a peptidomimetic was impractical. Based on these considerations, investigators modified the structure of GHRP-6 and identified more potent peptides (4–6, 18). For example, activity was enhanced by replacing D-Trp2 by D-2-(2-napthyl)alanine and His by D-alanine to furnish GHRP-2 (D-Ala-D-2 Nal-Ala-Trp-D-Phe-Lys-NH_2) (5). However, the peptides still had low oral bioavailability.

The development of the GHRPs had primarily emerged from *in vivo* studies and before attempting to identify peptidomimetics, it was essential to understand the cellular mechanisms involved in the action of GHRP-6. In 1989, the signal transduction pathway activated by GHRP-6 was reported (19). It was established that GHRP-6 acted directly on somatotrophs to cause GH release and to potentiate the effects of GHRH. In contrast to GHRH, which increases cAMP in somatotrophs, GHRP-6 alone had no effect on intracellular cAMP, but when combined with GHRH, the hexapeptide amplified the effects of GHRH on cAMP production (19). GHRP-6 was subsequently shown to activate L-type Ca^{2+} channels, to depolarize the plasma membrane of somatotrophs by inhibiting K^+ channels, and behave as a functional antagonist of somatostatin (8, 20–22). In contrast to GHRH, which stimulates GH release through the kinase A pathway, GHRP-6 apparently transduced its signal through protein kinase C (23). Phloretin, an inhibitor of protein kinase C, inhibited GHRP-6-stimulated GH release (23). Also, prolonged exposure of pituitary cells to phorbol esters before GHRP-6 treatment markedly attenuated the action of GHRP-6 without affecting GH release induced by GHRH (23). Subsequent studies showed that GHRP-6 stimulated IP_3 turnover, activated protein kinase C, and caused the release of intracellular stores of Ca^{2+} (24–26). Collectively, these data provided evidence that GHRP-6 acted through a receptor distinct from that of the GHRH receptor and were consistent with the notion that the GHRP-6 receptor was G-protein coupled.

To further evaluate differences between GHRH and GHRP-6 receptors, the kinetics of desensitization and resensitization of pituitary cells exposed to GHRP-6 and GHRH (27) were compared. These studies clearly showed that pituitary cells were desensitized very rapidly by GHRP-6 compared with GHRH, and that complete resensitization required interruption of exposure to GHRP-6 for at least 1 h (27). Having established the kinetics of desensitization, tachyphylaxis was deliberately induced to GHRH and GHRP-6 by prolonged continuous perifusion of each secretagogue. The cells were then challenged with GHRH and GHRP-6 to test for stimulation of GH secretion. Cross-desensitization was not evident, confirming that discrete receptors for GHRH and GHRP-6 were involved in the GH release pathways (27). The rapid rate of tachyphylaxis observed with GHRP-6 explained why some early studies failed to demonstrate an effect on GH release. In contrast to studies with the longer acting GHRH, where increases in GH in the medium of cultured pituitary cells are generally measured over 2–3 h, GHRP-6 culture medium must be sampled within 10–15 min of treatment for optimal results.

B. In vitro assays

Primary cultures of rat pituitary cells were used to screen for small molecules that selectively caused GH release. Because it was impractical to efficiently screen thousands of compounds in a primary cell assay, the structural classes to be assayed were rationally selected based on the key structural features of GHRP-6. However, to maximize the probability of finding a small molecule GH secretagogue, assay conditions were chosen that would identify compounds acting on GHRP-6, GHRH, or ion channel pathways (28). Selectivity for activation of somatotrophs, rather than mammotrophs or corticotrophs, was determined by assaying the pituitary cell culture medium for PRL and ACTH as well as GH.

The mechanism through which each active compound stimulated GH release was ascertained. For example, each compound was evaluated to determine whether it caused increases in cAMP or whether the protein kinase C pathway was involved in signal transduction. The actives were also assayed in the presence of specific GHRH and GHRP-6 antagonists to determine whether they were GHRH or GHRP-6 mimetics (29). Their effects on Ca^{2+}, K^+, and Na^+ channels and on membrane potential were also investigated. Finally, after identification of somatotrophs with the reverse hemolytic plaque assay (30), electrophysiology and Ca^{2+} imaging studies were applied to more carefully determine effects on the target cell (29). Having established the signal transduction pathway involved, pituitary cells were treated with each active compound in combination with GHRH or GHRP-6 to test for amplification of the respective pathways.

III. Molecular Design by Medicinal Chemistry

A. Benzolactams and L-692,429

Based on the structure of GHRP-6, and the knowledge that benzodiazepine and related templates mimic small peptides, approximately 120 compounds were selected for their ability

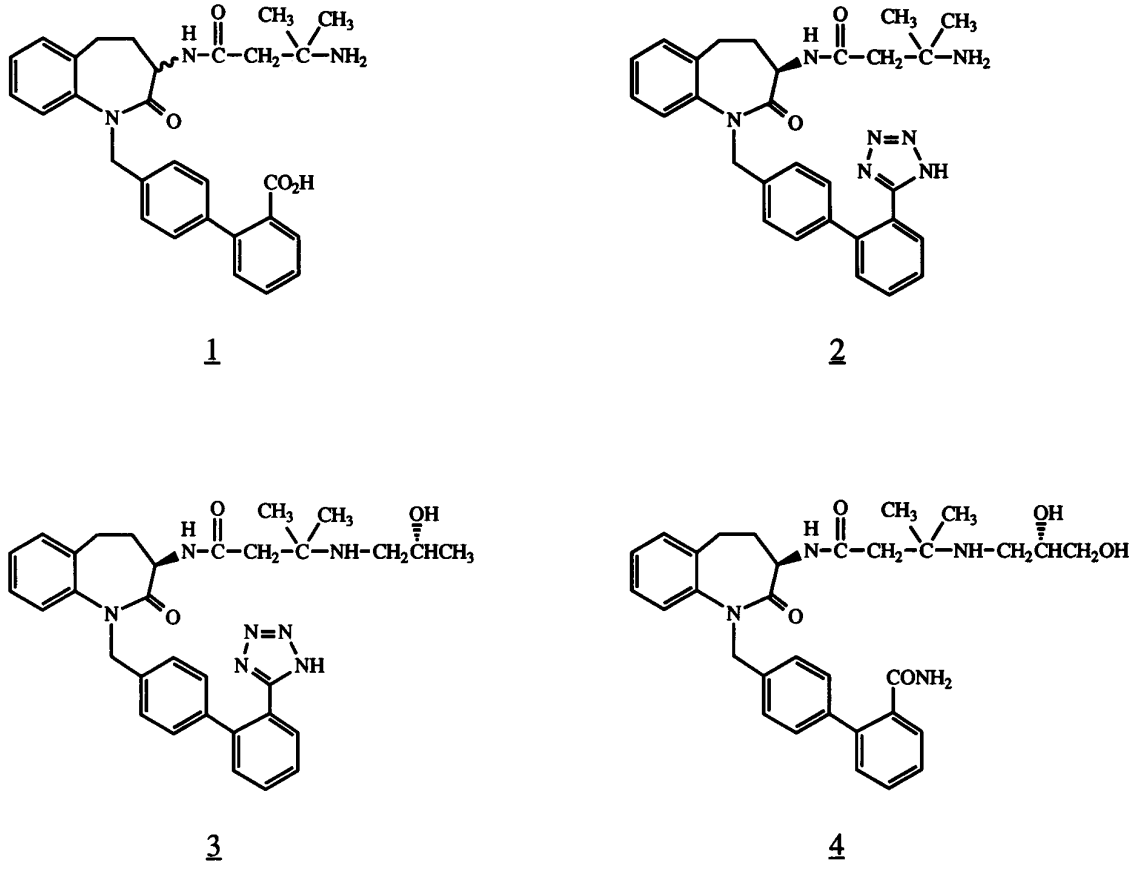

FIG. 1. Benzolactam GH secretagogues

to stimulate GH release. A benzolactam *1* (Fig. 1) with an EC_{50} of 3 μM for GH release in the rat pituitary cell assay was identified (29, 31). Replacement of the carboxylic acid moiety with a tetrazole and resolution of the enantiomers led to the discovery of L-692,429 (Fig. 1, compound *2*), a nonpeptide GH secretagogue with an EC_{50} of 60 nM (29, 31–33). L-692,429 was the first example of a potent nonpeptide GH secretagogue (29). L-692,428, the *S* enantiomer of L-692,429, was inactive, demonstrating the stereoselectivity of the response (Fig. 2). Using fluorescent ratio imaging, L-692,429 was shown to increase free intracellular Ca^{2+} in somatotrophs (Fig. 3); L-692,428 was ineffective (29). A structurally related nonpeptide antagonist of L-692,429 was identified and used in conjunction with peptide antagonists of GHRP-6 and GHRH to define the receptor target of L-692,429 (29). L-692,429 synergized with GHRH but, in the presence of a maximally effective concentration of GHRP-6, exhibited no additive effect on stimulating GH release from rat pituitary cells. The activity of L-692,429 was blocked by both peptide and nonpeptide antagonists of GHRP-6, but not by an antagonist of GHRH (29). These results were consistent with the notion that L-692,429 was a peptidomimetic of GHRP-6.

The specificity of L-692,429 was evaluated in rat pituitary cells to determine whether L-692,429 stimulated corticotrophs and lactotrophs in addition to somatotrophs. ACTH secretion was not affected; however, small increases in PRL were observed in some instances (34). The increase in PRL was more pronounced in pituitary cells derived from lactating female rats, suggesting that L-692,429 might be acting on somatomammotrophs (34–36). In contrast to the effects of L-692,429, when GnRH, CRF, and TRH were used as controls, profound increases in LH, ACTH, and PRL were measured in the pituitary cell culture medium; however, no clear synergism between L-692,429 and GnRH, CRF, or TRH was evident (K. Cheng and R. G. Smith, unpublished observations). To further investigate whether the effects on PRL release might be explained by activity on somatomammotrophs (35, 36), pituitary cells were treated sequentially with L-692,429 and TRH and monitored for changes in Ca^{2+} flux by fluorescent ratio imaging. Approximately 7% of the cells responded to both L-692,429 and TRH, indicating that somatomammotrophs as well as somatotrophs respond to L-692,429 (S.-S. Pong and R. G. Smith, unpublished results).

Attempts were made to improve the efficacy of L-692,429. A comparison of the biological activity of a series of six and eight-member lactam analogs of L-692,429 showed that the seven-member ring was preferred (31). Substitution with heterocyclic analogs of the benzolactam nucleus resulted in diminished activity (37). Continued exploration of structures related to L-692,429, focusing on refining structure-activity relationships in the amino acid side chain, revealed that the basic amine was an essential pharmacophore for GH-releasing activity (38). A systematic investigation of this dimethyl-β-alanine side chain led to the identification of L-692,585, a 2-(**R**)-hydroxypropyl analog (Fig. 1, compound *3*), which was 20-fold more potent than L-692,429 (38, 39). Comparison

of the binding data for L-692,585 [inhibition constant (K_i) = 0.8 nM] *vs.* L-692,429 (K_i = 63 nM) strongly suggested that the 2-hydroxypropyl moiety in L-692,585 makes an additional binding interaction with the GH secretagogue receptor. Although L-692,585 had much improved potency, and subsequent studies in beagle dogs showed it had highly reproducible oral activity, its oral bioavailability was unacceptably low (~4%) for clinical development. Replacement of the central phenyl ring of the biphenyl moiety in L-692,429 and L-692,585 was evaluated. A cyclohexenyl analog of L-692,585 showed similar activity to its parent, showing that the aromaticity of the central ring was not critical for bioactivity and suggested that this ring may serve to orient the benzolactam and phenyltetrazole pharmacophore (40). However, this structural change provided no improvement in oral bioavailability.

To investigate replacements for the 2′-tetrazole moiety of L-692,429, a variety of 2′-carboxamides and 2′-biphenyl analogs were evaluated. A 2′-carboxamide and *N*-2-hyroxypropyl tetrazole were found to have similar potency to the acidic tetrazole; however, *N*-alkyl tetrazoles, sulfonamides, and acyl sulfonamides were generally less potent replacements (41, 42). The primary and secondary carboxamides were potent GH secretagogues, and L-700,653 (Fig. 1, compound *4*) had improved oral bioavailability in dogs and in swine (42, 43). However, in spite of excellent potency, selectivity, and tolerability in animals, the relatively low bioavailability remained an issue with the benzolactam structural class; hence a different structural lead was sought (44–46).

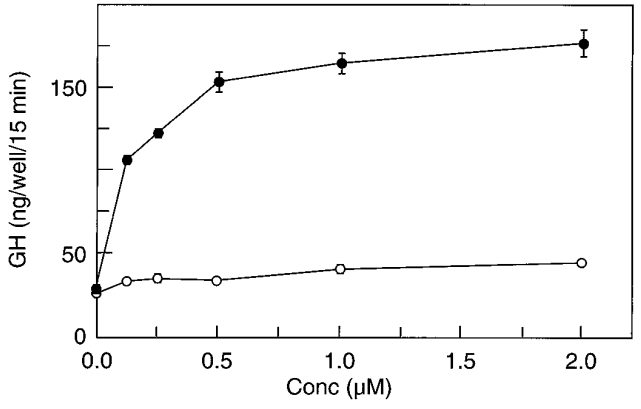

FIG. 2. Stereoselectivity of a nonpeptide GH secretagogue. A comparison of the effects of L-692,429 (R-enantiomer), ●, and L-692,428 (S-enantiomer), ○, on inducing GH release from cultured rat pituitary cells (29). [Reprinted with permission from R. G. Smith *et al.*: *Science* 260:1640–1643 (29). © 1993 American Association for the Advancement of Science.]

B. Spiroindanes and MK-0677

A new structural class of GH secretagogues was discovered by screening compounds from a project to prepare derivatized privileged structures for broad testing in receptor assays. The term "privileged structures" refers to structural units that are found on a recurring basis in receptor ligands. Their recognition and derivatization have been proposed as a useful way to prepare receptor agonists and antagonists (47). In the current instance, the successful strategy was to derivatize a spiroindanylpiperidine with capped amino acids. This piperidine derivative was considered a privileged structure since it was present in σ- (48) and oxytocin receptor ligands (49) and, in fact, was also present in a camphor sulfonamide lead *1* (L-368, 112, Fig. 4), which showed weak GH secretagogue activity (50). Thus, it could be inferred that the spiroindanylpiperidine would be an appropriate core from which to elaborate ligands for the putative GH secretagogue receptor. Its derivatization afforded L-252,564 (Fig. 4, compound *2*) whose activity as a secretagogue (EC_{50} = 50 nM) was remarkable since it was an unseparated mixture of four diastereoisomers. The other components of compound *2* are tryptophan and a quinuclidinylurea. To account for the high activity of this lead, it was noted that the quinuclidene group was also present in an unpublished Merck GH secretagogue lead, and tryptophan is a key amino acid in the GHRP-6 structure (50).

Although compound *2* was not orally active in dogs at 5

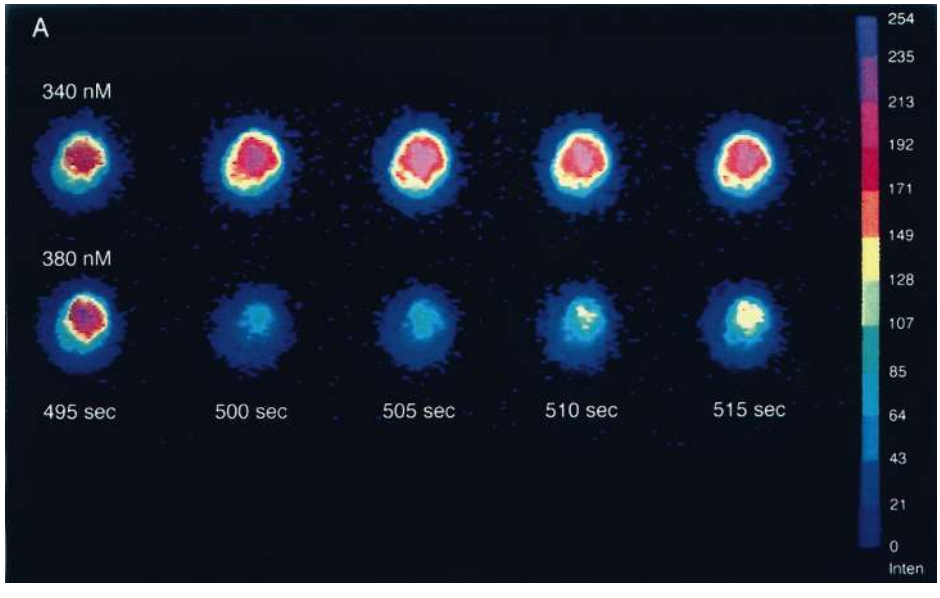

FIG. 3. Fluorescent ratio imaging showing the effect of L-692,429 on cytoplasmic free Ca^{2+} in a rat somatotroph. Images of a somatotroph at 340 nm and 380 nm are shown as a function of time after addition of L-692,429. The concentration of L-692,429 selected was 33-fold the EC_{50} for GH release, and the free intracellular Ca^{2+} increased from approximately 100 to 780 nM (29). [Reprinted with permission from R. G. Smith *et al.*: *Science* 260:1640–1643 (29). © 1993 American Association for the Advancement of Science.]

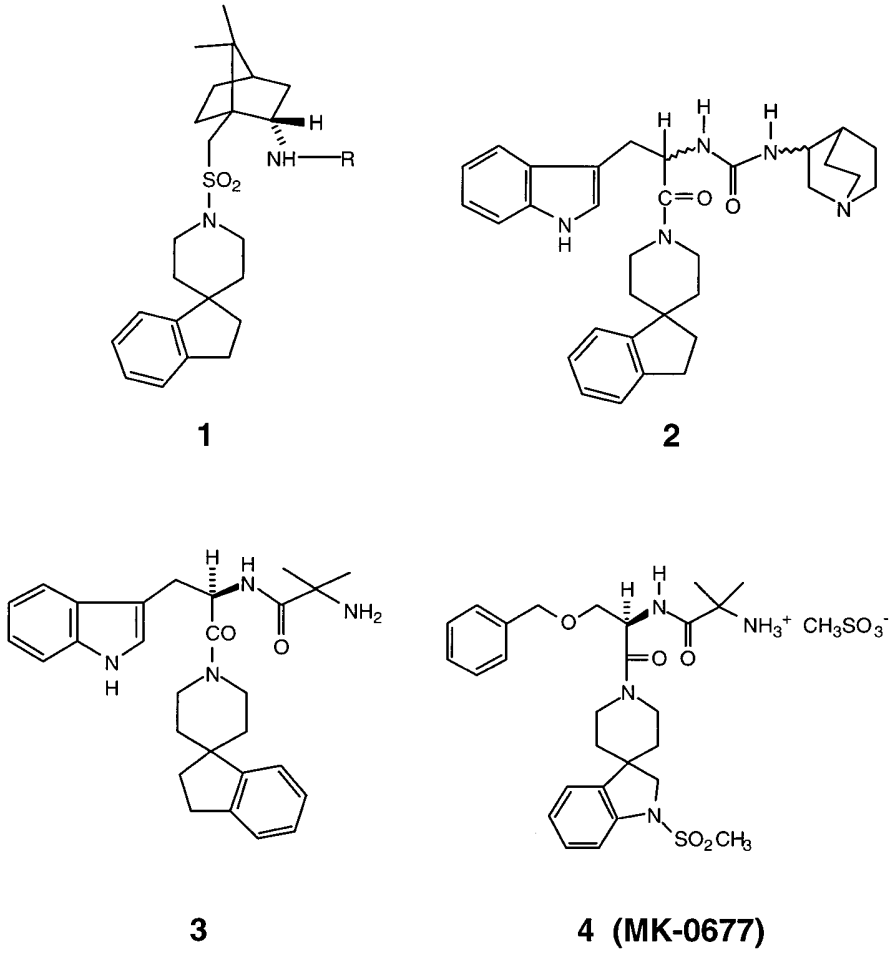

FIG. 4. Spiroindane GH secretagogues

mg/kg, good oral bioavailability was achieved by replacing the quinuclidinylurea with one of the amino acid side chains that had been discovered earlier in the benzolactam program. The bioavailability of this analog *3* (L-162,752) in dogs was more than 40% (51). Also, its selectivity for GH release was exemplified by IC_{50} values that were greater than 1 μM in more than 24 *in vitro* assays (51). Specificity is sometimes difficult to achieve in privileged structure derivatives, and this property, coupled with excellent oral bioavailability, gave considerable impetus to the lead's development.

Potency enhancement was achieved by the introduction of a carbonyl or hydroxyl substituent at the indane benzylic position. The oral activity of these compounds was disappointing; however, it was restored by the introduction of a methanesulfonamide group in this position. In addition, replacement of D-Trp by *O*-benzyl-D-serine further improved oral bioavailability. The resultant compound *4* (L-163,191) was active in the rat pituitary cell assay (EC_{50} = 1.3 nM) and was a specific GH secretagogue when counterscreened in more than 50 *in vitro* assays (50). These included oxytocin, enkephalin, cholinergic, adrenergic, serotonin, neurokinin, and galanin receptors. Its pharmacokinetic properties in rats included an oral bioavailability of between 6–22% (52), and in beagle dogs its bioavailability was greater than 60% with a terminal half-life of between 5–6 h (50, 52). In beagles, after oral administration of 1 mg/kg, GH was elevated for more than 6 h (50, 53). Insulin-like growth factor I (IGF-I) levels were also increased significantly. Most importantly, L-163,191 was the first GH secretagogue in its class demonstrated to provide a sustained increase in IGF-I levels for up to 24 h after a single oral dose (53, 54). Based on these properties, L-163,191 entered safety studies and then clinical studies as MK-0677. A series of analogs was also prepared (55, 56) to evaluate more extensively interactions with the MK-0677 receptor using site-directed mutagenesis studies.

C. Isonipecotic acid peptidomimetics

The Genentech group used a multidisciplinary approach in their discovery of a new series of small molecule GH secretagogues (Fig. 5). Toward the goal of determining the topographical requirements for the GH-releasing activity of the GHRPs, G-7203 (EC_{50} = 0.43 ± 0.11 nM), a cyclic analog of the linear hexapeptide GHRP-2, was developed (57, 58). Nuclear magnetic resonance studies showed that G-7203 was structured in water. Furthermore, the D-2-Nal-Ala-Trp-D-Phe fragment adopts a compact conformation with nested hairpin turns initiated at D-Lys1 and Ala3. Other less active cyclic GHRP-2 analogs did not readily adopt this conformation, suggesting that a precise arrangement of the three aromatic side chains was crucial for GH-releasing activity.

Peptidomimetic GH secretagogue G-7502 (EC_{50} = 10.6 ±

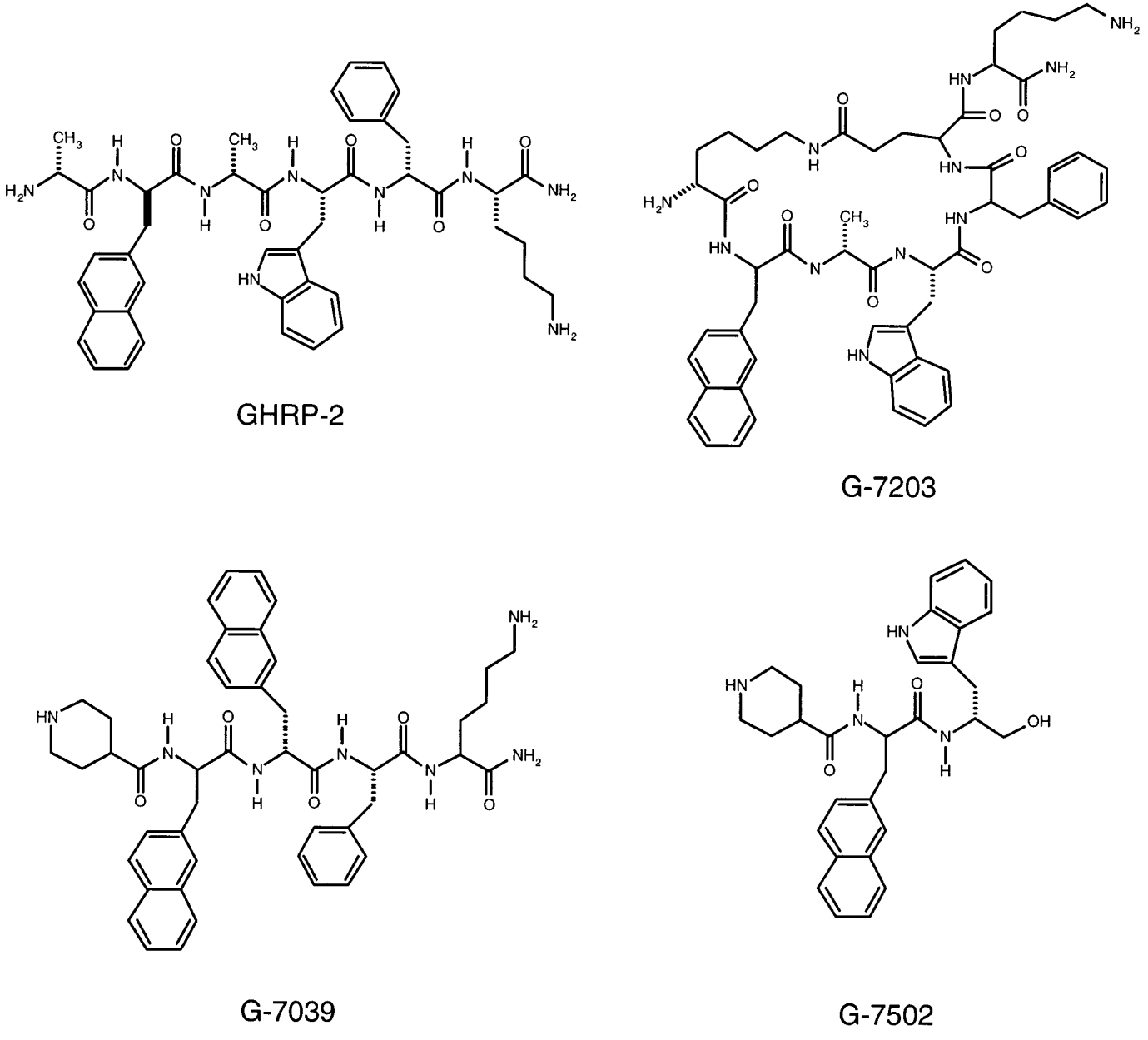

FIG. 5. GHRP-2 and Genentech GH secretagogues

6.2 nM) was developed from extensive medicinal chemistry studies on GHRP-6 (57, 58). An interesting finding from their studies leading to the discovery of G-7039 (EC_{50} = 0.18 ± 0.04 nM) was that the D-Nal-Ala-Trp-D-Phe triaromatic core of GHRP-6 could be presented as D-2-Nal-D-2-Nal-Phe. Furthermore, an isonipecotic acid amino side chain was identified as a satisfactory replacement for the N-terminal histidine of GHRP-6. Further optimization of G-7039 by removal of the Phe residue led to a series of small molecule GH secretagogues exemplified by G-7502. These studies lend further support to proposals made by the Merck group that the essential GH secretagogue pharmacophore is comprised of only a diaromatic core and a basic amine. All of the Genentech GH secretagogues, including G-7039 and G-7502, released GH from rat primary pituitary cells in a dose-dependent manner, synergized with GHRH but not GHRP-6, and demonstrated homologous desensitization after prolonged exposure. Furthermore, coincubation with somatostatin completely suppressed the GH-releasing activity of these compounds. At 100 nM concentration, G-7030 and G-7502 were relatively specific for releasing GH except for small increases in PRL. These results suggest that these new peptidomimetics exert their action through the GHRP pathway (58).

IV. Characterization of the MK-0677 Receptor

A. Pituitary gland

Based on binding studies using [^{3}H]naloxone as a ligand, it was clear that although GHRP-6 and its analogs were

derived from met-enkephalin, neither GHRP-6 nor the peptidomimetics bind to opiate receptors. Moreover, their GH-releasing activity was not blocked by coadministration of naloxone (34). Extensive evaluation of L-692,429 and MK-0677 showed that they lacked activity on different opiate receptor subtypes and in more than 50 different receptor assays (29, 50, 59). Attempts to define a receptor in the pituitary gland using radiolabeled GHRP-6 failed to show a correlation between high-affinity, low-capacity binding and biological activity of both peptide and peptidomimetic GH secretagogues (60).

To characterize the MK-0677 receptor, high-specific activity radiolabeled MK-0677 (800–1100 Ci/mmol) was synthesized by substituting ^{35}S for ^{32}S in the molecule (60, 61). [^{35}S]MK-0677 bound with high affinity [dissociation constant (K_d) = 140 pM] and limited capacity (B_{max} = 6.4 fmol/mg protein) to porcine pituitary membranes (Fig. 6). This concentration of binding sites in pig pituitary is remarkably low, but in rat pituitary membranes the concentration is even lower (2 fmol/mg protein). [^{35}S]MK-0677 binding was displaced by L-692,429, L-692,585, and by the peptide GH secretagogue GHRP-6, but not by GHRH or somatostatin (60). The K_i values in the binding assay correlated with the EC_{50} values for stimulating GH release in the rat pituitary cell assay (Table 1). Consistent with [^{35}S]MK-0677 binding to a G protein-coupled receptor, binding was dependent upon Mg^{2+} (5 mM) and displaced by GTP-γ-S (10 nM) but not by ATP-γ-S (59, 60). Remarkably, competition binding studies with L-692,429 and GHRP-6 showed that these two structurally distinct molecules were competitive inhibitors of MK-0677 binding (59). By contrast, as anticipated for a G protein-coupled receptor, GTP-γ-S was an allosteric inhibitor (59, 60). To further explore the specificity of [^{35}S]MK-0677 binding, met-enkephalin, GnRH, TRH, galanin, gastrin releasing peptide, substance P, MSH, isoproterenol, dopamine, bromocriptine, propanolol, and clonidene were tested in the binding assay at a concentration of 1 μM; none competed for [^{35}S]MK-0677 binding (60).

Digitonin was used to solubilize the MK-0677 receptor from porcine anterior pituitary membranes. The receptor was recovered as a receptor-[^{35}S]MK-0677-G protein complex. The apparent molecular mass of the complex determined by gel filtration under native conditions was 255 kDa (62). Attempts to isolate the receptor before labeling with MK-0677 were unsuccessful. Treatment of the solubilized receptor in the digitonin micelle with GTP-γ-S caused dissociation of MK-0677 from the receptor with an EC_{50} of 5 nM; ATP-γ-S was ineffective even at 10 μM, consistent with the MK-0677 receptor being coupled to a G protein in the soluble complex (62).

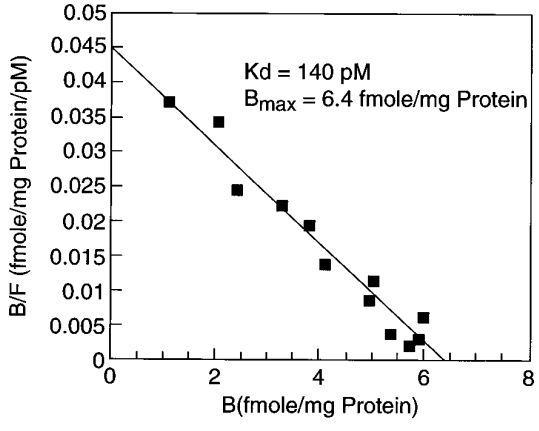

FIG. 6. Scatchard analysis of [^{35}S]MK-0677 binding to pig pituitary membrane showing a single class of high-affinity binding sites (60). [Reproduced with permission from S.-S. Pong *et al.*: *Mol Endocrinol* 10:57–61, 1996 (60). © The Endocrine Society.]

TABLE 1. Specificity of [^{35}S]MK-0677 binding to rat pituitary membranes and correlation with GH-releasing activity on rat pituitary cells (60)

	Concentration (nM)	
	K_i (binding)	EC_{50} (secretion)
MK-0677	0.24	1.3
L-692,429 (MK-0751)	63.0	60.0
L-692,428 (inactive stereoisomer)	>5000.0	>5000.0
L-692,585 (more active benzolactam)	0.8	3.0
GHRP-6	6.0	10.0

[Adapted with permission from S.-S. Pong *et al.*: *Mol Endocrinol* 10:57–61, 1996 (60). © The Endocrine Society.]

To determine whether the binding to pituitary membranes was localized to somatotrophs, a biotinylated analog of MK-0677, L-164,683, was prepared as a suitable ligand for immunofluorescence studies (63). L-164,683 was an excellent competitor for [^{35}S]MK-0677 binding (IC_{50} = 0.2 nM) and stimulated GH release with an EC_{50} of 2.5 nM. Primary cultures of rat pituitary cells were treated with L-164,683 for 3 min at 37 C and treated with avidin-Texas Red. GH-containing cells were labeled with fluorescein-conjugated goat anti-rabbit IgG. Dual fluorophor labeling for GH and the MK-0677 receptor by confocal microscopy showed that about half of the GH-containing cells also expressed the MK-0677 receptor (Fig. 7). L-164,683 binding was confined to cells that contained GH (63). While only about half of the GH-containing cells showed binding of the MK-0677 analog, based on limits of sensitivity of the assay, this proportion should be considered a low estimate. More quantitative estimates have been attempted by localization using MK-0677 receptor antibodies, and preliminary results suggest that all GH-containing cells in the pituitary gland express the MK-0677 receptor (R. G. Smith, A. D. Howard, S. D. Feighner, and J. W. Woods, unpublished results).

B. Hypothalamus

Hypothalamic membranes were isolated from rats to test for the presence of the receptor in the hypothalamus. [^{35}S]MK-0677 bound with high affinity (K_d = 170 pM), and the concentration of binding sites (B_{max} = 8 fmol/mg protein) was higher than measured in rat anterior pituitary membranes (64). Binding to rat hypothalamic membranes was Mg^{2+} dependent and inhibited by nonhydrolyzable analogs of GTP such as GTP-γ-S and guanyl-imidodiphosphate (64). These properties are consistent with MK-0677 binding to a G protein-coupled receptor in the hypothalamus. Membranes isolated from liver, thalamus, cerebral cortex, medulla, pons, and posterior pituitary membranes were assayed, but high-affinity [^{35}S]MK-0677 binding was not de-

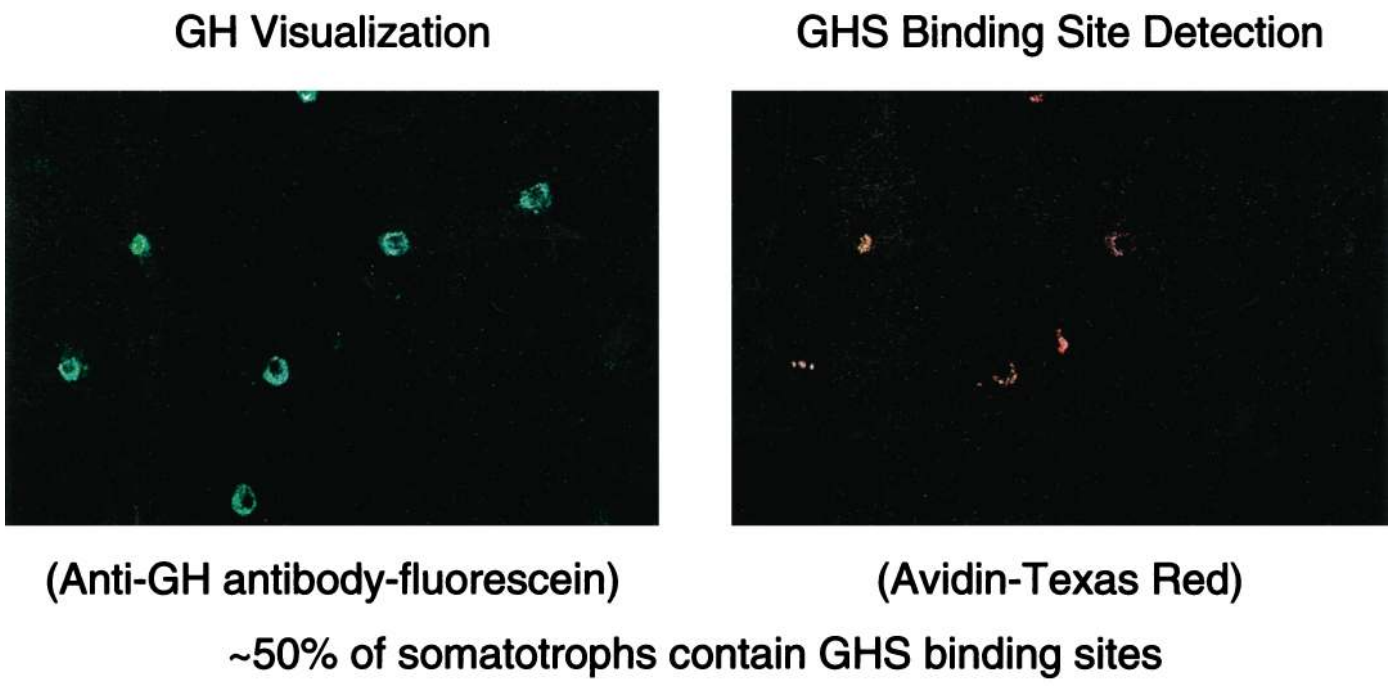

FIG. 7. Dual fluorophore localization of MK-0677-binding sites and GH in rat pituitary primary cultures (63). *Left panel*, Identification of rat GH with a rabbit anti-rat GH antibody and fluorescein-conjugated goat anti-rabbit antibodies. *Right panel*, Identification of GH secretagogue-binding sites on the same cells with biotinylated MK-0677 (L-164,683; 1 μM; 3 min 37 C) and avidin Texas red. Cells were first reacted with L-164,683 (15 min 4 C) and avidin, after which the cells were fixed and permeabilized and treated for GH detection. Note that approximately 50% somatotrophs are labeled for MK-0677-binding sites, and all cells labeled with the MK-0677 derivative contain GH.

tected, demonstrating tissue specificity of MK-0677 binding (64). In common with binding to rat anterior pituitary membranes, binding to hypothalamic membranes was highly selective for GHRP-6, GHRP-2, MK-0677, L-692,429, and L-692,585. Moreover, the relative IC_{50} values for displacement of [^{35}S]MK-0677 binding was highly correlated with activity in stimulating GH release from cultured rat pituitary cells (64). Thus the hypothalamic receptor has identical characteristics to the MK-0677 receptor identified in the anterior pituitary gland.

V. Signal Transduction Pathway

MK-0677, in common with L-692,429 but in contrast to GHRH, does not increase intracellular cAMP levels in pituitary cells (50, 59). However, in combination with GHRH, MK-0677 amplifies the GHRH-induced increase in cAMP and potentiates GH release (59). At doses of MK-0677 that maximally stimulated GH release *in vitro,* additional stimulation is not observed in the presence of maximally stimulating concentrations of L-692,429. Similar observations were made when GHRP-6 was substituted for L-692,429 indicating, in agreement with binding studies, that GHRP-6 and these peptidomimetics all mediate their effects through the same receptor. The synergistic effects of the GH secretagogues on the GHRH pathway were mimicked by phorbol myristic acetate. Preincubation of rat pituitary cells with phorbol myristic acetate for 24 h, before treatment with MK-0677, profoundly attenuated the stimulation of GH release by MK-0677 (50). These results, and the cation and nucleotide dependence of MK-0677 binding, suggested that MK-0677 interacts with a G-protein coupled receptor that activates phospholipase C (59, 60). Consistent with activation of this pathway were the earlier observations that other ligands for this receptor, such as L-692,429 and GHRP-6, increase IP_3 turnover (65), increase free intracellular Ca^{2+} through an IP_3 pathway, and cause translocation of protein kinase C (24–26, 65).

The role of L-type Ca^{2+} channels in the transduction pathway was confirmed by fluorescence ratio imaging in somatotrophs after treatment with either L-692,429 or MK-0677 (29, 59). Nifedipine and ω-agatoxin IIIA, but not conotoxin, were shown to block increases in intracellular Ca^{2+}, consistent with activation of L-type Ca^{2+} channels (66–68). Electrophysiology studies showed that the GHRP-6 peptidomimetics blocked K^+ currents in somatotrophs, resulting in depolarization and electrical spiking to enhance Ca^{2+} entry through voltage gate channels (21, 69, 70). Modulation of these channels was evident when perforated patch clamp or on-cell single-channel recording techniques were used, but not when the cells were dialyzed using the whole cell voltage clamp configuration, suggesting that a soluble second messenger was involved. The depolarizing effects were confirmed using the membrane-sensitive dye bisoxanol (29). Depolarizing agents such as the potassium channel blockers tetraethylammonium and 4-amino pyridine and the sodium channel agonist veratridine had no additive effects on GH secretion induced by the peptidomimetics; rather, they mimicked the peptidomimetics by amplifying the effects of GHRH (8). The fact that the peptidomimetics cause depolarization explains their functional antagonism of somatostatin, since somatostatin hyperpolarizes somatotrophs by in-

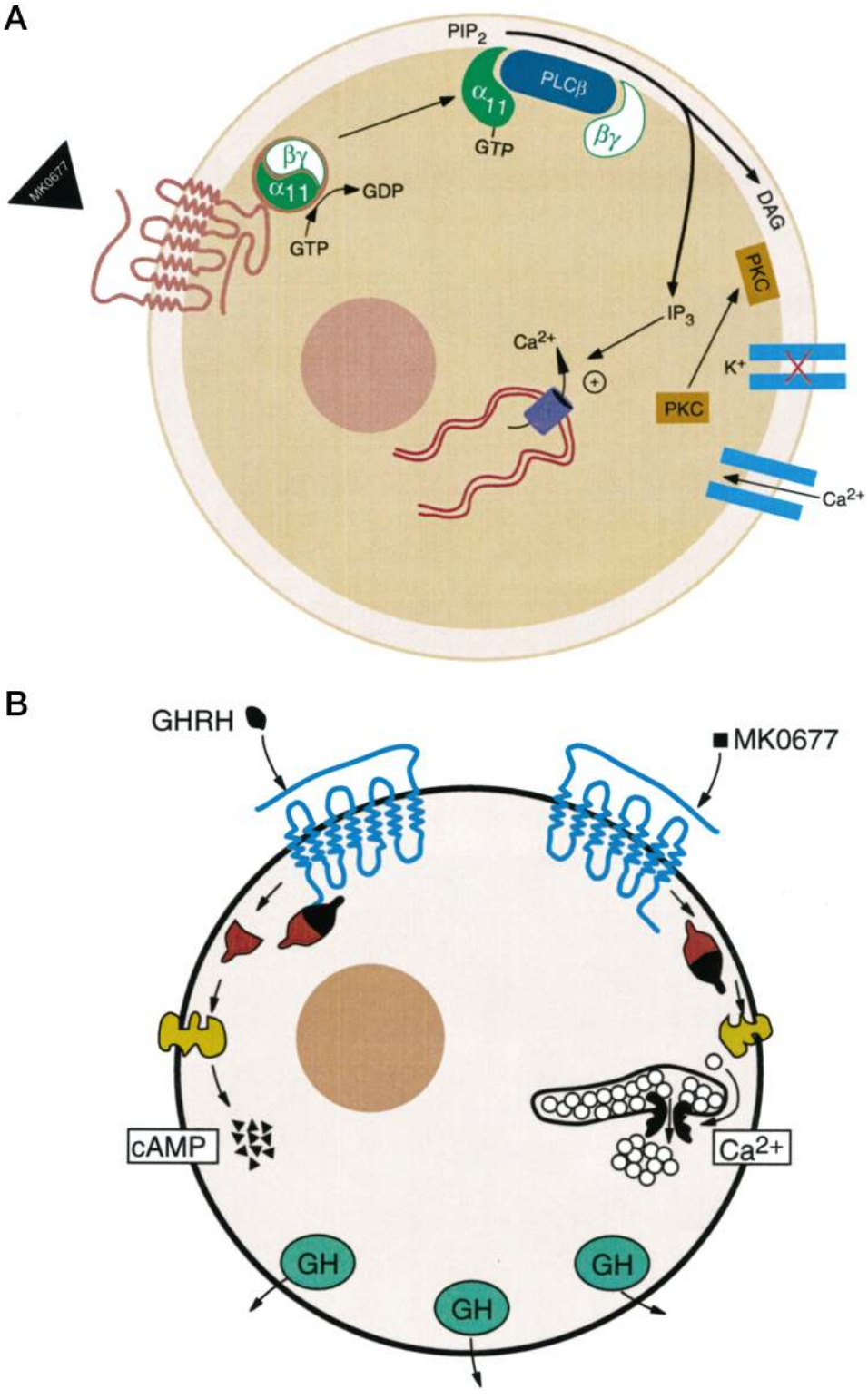

FIG. 8. A, Signal transduction pathway activated by ligands that interact with the MK-0677 receptor. B, GHRH and MK-0677 act on discrete receptors and transduce their signal through different pathways.

creasing potassium conductance (71). The magnitude of GH release caused by depolarizing agents alone is less than that induced by the peptidomimetics, emphasizing the importance of other aspects of the signal transduction pathway. The MK-0677 receptor via phospholipase C activates Ca^{2+} and inhibits K^+ channels to facilitate GH secretion (Fig. 8A). The IP_3-mediated redistribution of intracellular Ca^{2+} alone does not explain all the effects of these secretagogues because somatostatin does not prevent the Ca^{2+} redistribution but does inhibit GH release (30); rather, it is speculated that IP_3 facilitates GH release by synchronizing docking of GH-secretory granules to the plasma membrane (72, 73).

The GHRH receptor is also G protein coupled (74) but activates a different signal transduction pathway to that of the MK-0677 receptor. These differences are illustrated in Fig. 8B. GHRH stimulates adenylate cyclase, resulting in an increase in cAMP (75), whereas GHRP-6, L-692,429, and MK-0677 activate phospholipase C (23, 25, 26, 59, 65). Apparently, through cross-talk in the signal transduction pathways, these ligands synergize with GHRH to further increase intracellular cAMP (8, 59). This augmentation might be mediated by interactions between the $G_{\beta\gamma}$ subunits associated with the MK-0677 receptor and $G_{\alpha s}$ of the GHRH receptor complex (76). A potential bonus of the synergy of GHRH and MK-0677 is that increases in cAMP have been associated with increased GH synthesis (77). Based on a knowledge of the signal transduction pathway that results in amplifying the activity of GHRH and functionally antagonizing somatostatin, it becomes clear why GHRP-6 and the peptidomimetics are so effective in inducing GH release *in vivo*. The intriguing properties of these synthetic GH secretagogues force us to speculate that such ideal characteristics are shared by an undiscovered natural hormone that plays a key role in the physiological regulation of pulsatile GH release.

VI. Cloning the GH Secretagogue Receptor

Having established from binding data and biochemical studies that MK-0677 probably binds to a G protein-coupled receptor that signals through phospholipase C, an expression-cloning strategy was developed for the MK-0677 receptor by microinjecting size-fractionated poly A^+ RNA from pig pituitaries into *Xenopus* oocytes (16). Expression of the RNA from one fraction resulted in an MK-0677-induced calcium activated chloride current, but the assay reproducibility was low. A more robust cloning strategy was developed, which coupled receptor expression in *Xenopus* oocytes to a functional endpoint that measured MK-0677-induced Ca^{2+} release (16). The Ca^{2+}-sensitive bioluminescent reporter protein aequorin was used successfully as a signal for positive coupling, but responses to MK-0677 were still weak and variable. It was speculated that expression of a specific G protein essential for receptor coupling might be limiting; therefore, cRNAs encoding a series of G proteins ($G\alpha_{11}$, $G\alpha_q$, $G\alpha_{16}$, $G\alpha_{13}$, $G\alpha i_1$, $G\alpha i_3$, $G\alpha_o$) were individually coinjected into *Xenopus* oocytes together with pituitary gland poly A^+ RNA and aequorin cRNA. Only $G\alpha_{11}$ provided a highly reproducible Ca^{2+}-mediated luminescence signal in response to MK-0677 (16). This robust expression system was used to screen pools of cRNA from a pig pituitary gland cDNA library for an MK-0677-inducible signal. Approximately 2×10^6 individual cDNAs from a pig pituitary library were screened in pools of 10,000. Stepwise fractionation of a single positive pool resulted in the isolation of a single cDNA clone that conferred both MK-0677-activated aequorin luminescence and an inward chloride current in *Xenopus* oocytes. Interestingly, supplementation with $G\alpha_{11}$ was unnecessary when cRNA pool complexity dropped below 50 clones.

The nucleotide sequence of the full-length swine MK-0677 receptor cDNA-1a predicted a protein of 366 amino acids with seven transmembrane (7-TM)-spanning domains, three intra- and extracellular loops, and a G protein-coupled receptor triplet signature sequence (16). The human receptor was subsequently cloned, and its predicted topology is represented in Fig. 9. Genomic analysis by Southern blotting was consistent with a single highly conserved gene in human, chimpanzee, bovine, rat, and mouse (16). Sequence alignments showed that the swine MK-0677 receptor was 93% identical and 98% similar at the amino acid level to the human receptor (Fig. 10). Additional cDNAs clones were obtained from pig and human libraries that encode a shorter form of the MK-0677 receptor (16). Receptor 1b cDNA encodes a polypeptide of 289 amino acids that lacks transmembrane domains 6 and 7 of the 1a receptor (16). This truncated receptor is identical to the 1a receptor from the translation initiation codon to Leu-265 beyond which the cDNA is fused to a short contiguous reading frame of 24 amino acids followed by a translation stop codon. This 24-amino acid sequence is highly conserved in both the pig and human MK-0677 receptor genes (16). A similarly truncated mRNA has been reported for the neuropeptide Y1 (NPY1) receptor (78). Inspection of the amino acid sequence of the human 1a receptor revealed a G protein-coupled receptor signature [ERY142], a series of potential N-glycosylation sites, protein kinase C, casein kinase II phosphorylation sites, a cAMP/cGMP-dependent phosphorylation site [346–349 RKLS], myristoylation sites, and an amidation site (235–238 IGRK).

The pharmacological properties of MK-0677 receptors 1a and 1b were investigated with functional assays using aequorin bioluminescence and electrophysiology in *Xenopus* oocytes (16). In addition, after transient transfection of HEK293 or COS cells with 1a and 1b cDNAs, aequorin bioluminescent assays (in HEK293 cells) and [^{35}S]MK-0677 competition binding assays were performed. Induction of a Ca^{2+}-activated Cl^- current in response to MK-0677 was observed in oocytes injected with 1a cRNA but not with 1b cRNA. Similarly, in COS-7 cells transiently expressing 1a or 1b, aequorin bioluminescence was only induced by MK-0677 with the 1a clone. Transfection of clone 1a into COS-7 cells resulted in high-affinity binding of [^{35}S]MK-0677 to plasma membranes. The binding was inhibited by GHRP-6 and GHRP-2 but not by GHRH, somatostatin, TRH, or galanin (Fig. 11). Because this receptor binds GHRPs in addition to MK-0677, it was designated the "GH secretagogue receptor" (GHS-R). In contrast to GHS-R1a, high-affinity saturable binding was not evident in membranes from cells expressing GHS-R1b cDNA. Although no direct function has yet been assigned to receptor 1b, it may play a regulatory role in the context of modifying the function of a related G protein-coupled receptor. For example, it has been shown that inactive truncated forms of related G protein-coupled receptors can be coexpressed to rescue their function (79).

The closest identities of the GHS-R with other related G protein-coupled receptors were to the neurotensin receptor (NT-R) and thyroid releasing hormone receptor (TRH-R) with ~35% and 29% identity, and 59% and 56% similarity, respectively (values are for the human GHS-R open reading frame compared with human NT-R and TRH-R). A dendrogram of related G protein-coupled receptors suggest that the GHS-R presents a new family of the NT-R, TRH-R branch of the phylogenetic tree (Fig. 12).

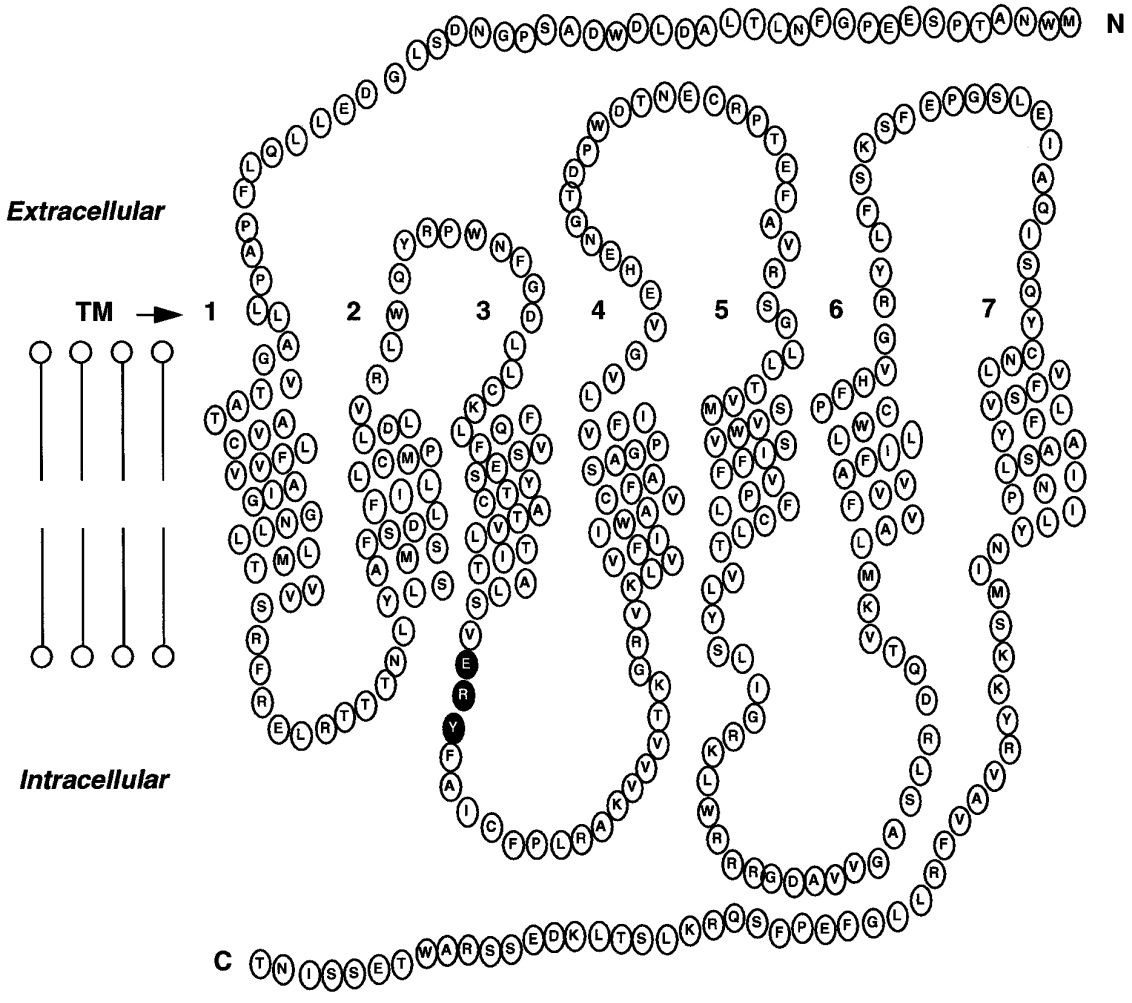

FIG. 9. Predicted membrane topology and amino acid sequence of the human type 1a GHS-R. TM denotes transmembrane domains 1–7.

It has been suggested from pharmacology studies that a subtype of the GHS-R may be expressed in the pituitary gland. For example, GHRP-2, in contrast to GHRP-6, has been reported to increase cAMP levels in cultured pituitary cells rather than activating phospholipase C (80). However, since both GHRP-2 and GHRP-6 bind with high affinity to GHS-R, these differences might be explained by each ligand conferring a different conformation on the GHS-R. As a consequence, the receptor could couple to different G proteins and hence signal through alternative signal transduction pathways. This behavior is illustrated by the octopamine/tyramine receptor, where the presence of a single hydroxyl group on the ligand results in differential coupling to different second messenger systems (81). Activation of alternative pathways would also depend on the relative concentration of specific G proteins. Therefore, although we cannot rule out the existence of different pituitary receptor subtypes for GHRPs being involved in the control of GH release, the experimental observations can be rationalized by the same receptor coupling to different G proteins.

A. Chromosomal localization

The human GHS-R was mapped by fluorescence *in situ* hybridization to band 3Q26.2 (82). Genes whose deficiencies affect GH release do not map to this region. Interestingly, however, this location is close to the map position reported for the Brachmann-de-Lange Syndrome, a pre- and postnatal growth deficiency (83–85). Mapping of the Brachmann-de-Lange Syndrome is based on chromosome duplication and translocation mutants, which always include region 3q26 (interval 3q26.31–q27.3). Given the close proximity between the GHS-R gene and the presumed Brachmann-de-Lange location, it will be important to determine whether these subjects respond to MK-0677 treatment and whether they have alterations in the gene encoding the GHS-R.

VII. Action of the Peptidomimetic GH Secretagogues in the Central Nervous System

The central effects of GHRP-6 and the peptidomimetics are inferred by the demonstration in sheep and guinea pigs that the doses required to cause GH release are at least 10- to

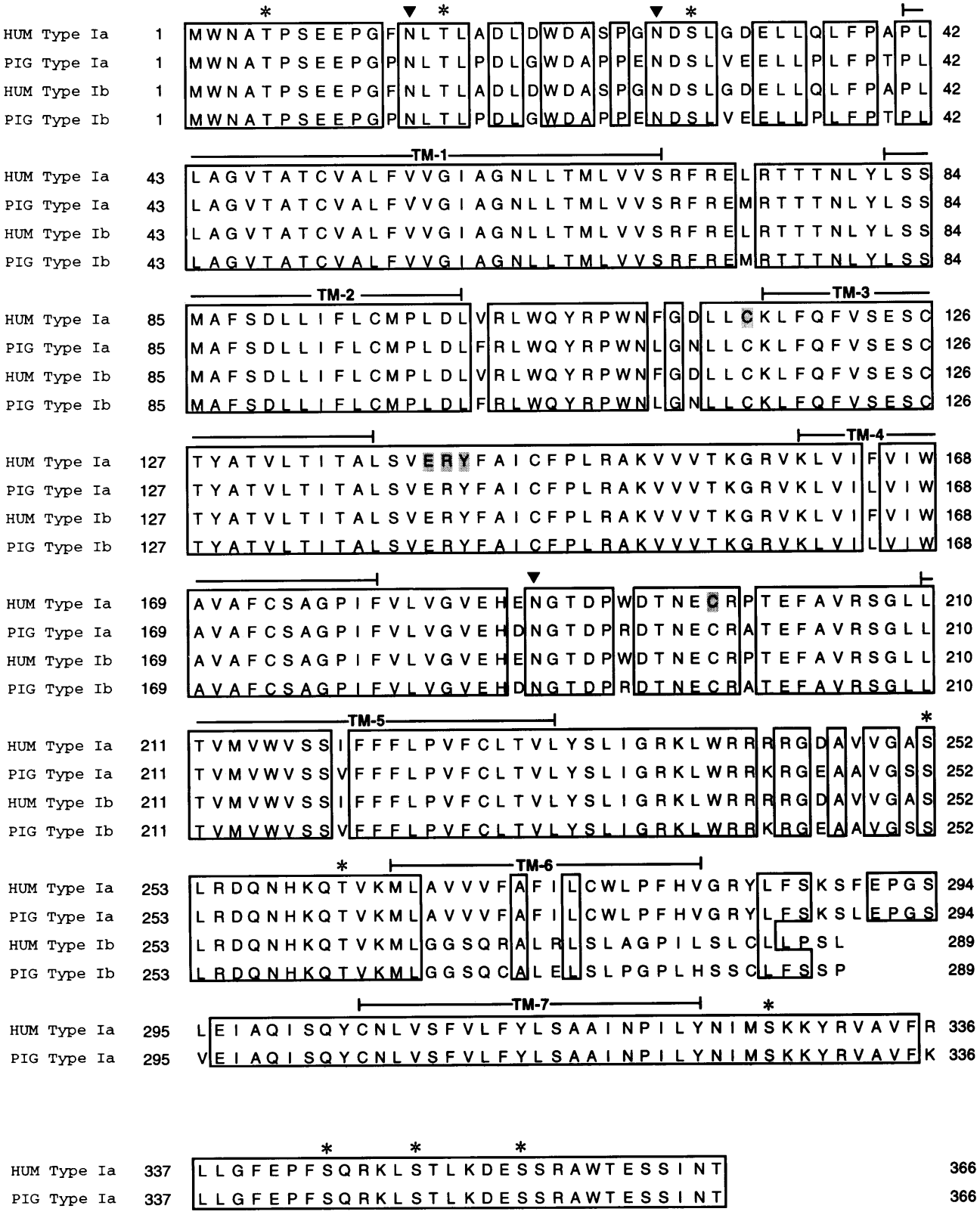

FIG. 10. Predicted amino acid sequences for the human and swine type 1a and 1b GHS-R. Identities are highlighted in the *boxed* amino acid sequences (16). Conserved cysteine residues and the GPC-R signature sequence (ERY142) are *shaded gray*. N-Linked glycosylation sites and potential phosphorylation sites are highlighted with *arrowheads* and *asterisks*, respectively. The transmembrane (TM) domains are *overlined* and numbered sequentially. The *numbers on the left* and *right* refer to the sequentially numbered amino acid residues (1–366 for the type 1a and 1–289 for the type 1b receptors). [Reprinted with permission from A. D. Howard *et al.*: *Science* 273:974–977 (16). © 1996 American Association for the Advancement of Science.]

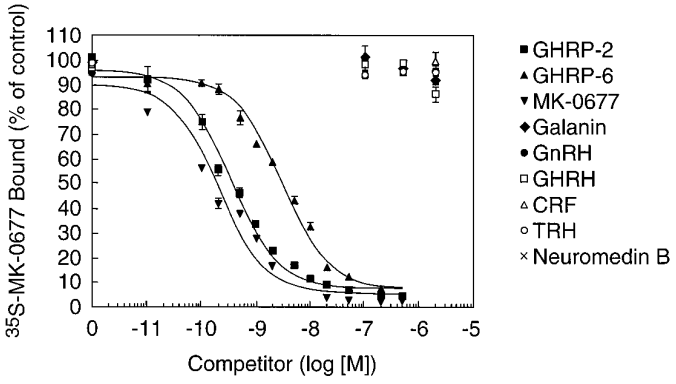

FIG. 11. Competition binding assay showing displacement of [^{35}S]MK-0677 (.24 nM) from COS cell membranes isolated from cells transiently expressing GHS-R1a. [Reprinted with permission from A. D. Howard *et al.*: *Science* 273:974–977 (16). © American Association for the Advancement of Science.]

100-fold lower when injected into the third ventricle than when the compounds are administered peripherally (86, 87). Intravenous administration of GHRP-6 and the peptidomimetics L-692,429, L-692,585, and MK-0677 into conscious rats and mice increased c-*fos* expression in the arcuate nucleus at concentrations consistent with their relative biological activites in releasing GH (86, 88–90). *In situ* hybridization histochemistry studies with *little (lit/lit)* mice and *dwarf* (*dw/dw)* mice treated with MK-0677 (Fig. 13) also showed pronounced increases in c-*fos* expression (89). *Lit/lit* mice have a reduced capacity to release GH because they lack a functional GHRH receptor (74, 91–93), and *dw/dw* mice lack GH (94, 95). Therefore, activation of *fos* by GHRP-6 and the peptidomimetics cannot be explained by indirect effects on GHRH receptors or through GH (86, 88, 89, 96), but rather as a direct consequence of a central action of GHS-R ligands. In rats treated with GHRP-6, approximately 25% of neurons showing an increase in c-*fos* activity express GHRH mRNA, and 51% express NPY mRNA (97). The activation of GHRH-containing neurons suggests that GHRP-6 and the peptidomimetic secretagogues stimulate the release of GHRH. Indeed, increases in GHRH have been measured in hypothalamic-pituitary portal vessels of sheep after systemic treatment with GHRPs (98, 99). The finding that GHRP-6 also activates NPY-containing neurons potentially explains the increased feeding behavior seen in rats treated with GHRP-6 (100) and the observed increases in corticosteroid and ACTH release (101, 102). Clearly, activation of Fos immunoactivity or c-*fos* mRNA expression should not be considered a direct and sole marker since other neurons in the brain that do not express Fos may be activated, and of course those expressing Fos might be activated indirectly as a result of derepression.

Experiments were designed to measure effects of GHRP-6 and the peptidomimetics on individual neurons projecting to the median eminence. Recordings from electrodes implanted in the arcuate nucleus of anesthetized rats showed that intravenous administration of GHRP-6 and L-692,585 stimulated electrical activity in secretory neurons that had been identified antidromically as projecting to the median eminence (88). Use of the retrograde tracer Fluorogold showed that 68–82% of cells excited by GHRP-6 project outside the blood-brain barrier (103). Interestingly, a subset of arcuate neurons that did not project to the median eminence was inhibited by GHRP-6 and L-692,585(88). Since previous studies have shown that somatostatin-containing cells in the arcuate nucleus also do not project to the median eminence, it is tempting to speculate that GHS-R ligands inhibit somatostatin release from these cells. Reducing somatostatin tone on GHRH neurons would facilitate GHRH release (104).

In situ hybridization studies using a cDNA probe selective for GHS-R 1a to sections of rhesus monkey and rat brains demonstrate that the receptor is expressed in the arcuate nucleus (16). Localization of expression in this area of the hypothalamus is consistent with electrophysiology experiments and c-*fos* expression, suggesting that these molecules act on GHRH-containing arcuate neurons (97). More complete localization studies have been completed in rat brain and pituitary gland and show that GHS-R is expressed in the anterior pituitary gland and in regions of the brain outside those generally considered to be involved in GH release (Fig. 14 and Ref. 105). Indeed, expression of GHS-R is seen in the anterior hypothalamus, suprachiasmatic nucleus, supraoptic nucleus, ventromedial hypothalamus, arcuate nucleus, dentate gyrus, tuberomamillary nucleus, pars compacta of substantia nigra, the ventral tegmental area, dorsal raphe nuclei, and median raphe nuclei (105).

The presence of GHS-R RNA in regions of the brain outside the arcuate nucleus is remarkable. The hippocampus is enriched with neurotransmitter systems and has been implicated in learning and memory (106, 107). The substantia nigra and ventral tegmental areas are main centers for dopaminergic cell bodies that are involved in many biological functions such as motor control and reinforcement behavior (108). The dorsal and median raphe nuclei are centers for serotonergic neurons that project to different parts of the central nervous system and are implicated in a variety of functions including nociception, affective behaviors, and feeding (109). Expression of the GHS-R in brain regions not generally associated with GH release is intriguing and suggests a broader physiological significance for the role of the natural ligand of the GHS-R.

VIII. Peptidomimetic GH Secretagogues *in Vivo*

A. Animal models

Experiments with cultured pituitary cells demonstrated that *in vitro* desensitization to the peptidomimetic GH secretagogues occurred rapidly. However, animal studies with a compound having a relatively long half-life suggested that the effects on GH release were sustained (43). In guinea pigs, when GH was monitored at 10-min intervals during a constant infusion of L-692,585, a sustained amplification of episodic GH release was observed, similar to that seen with GHRP-6 (7, 87). Intriguingly, L-692,585 initiated GH pulsatility, suggesting that this class of compounds has the capacity to reset the ultradian rhythm of GH release (87).

The action of the peptidomimetic GH secretagogues, L-692,429 and L-692,585 (5–100 μg/kg), on GH dynamics was evaluated in a crossover design with four male and four female beagle dogs (110, 111). Peak GH levels were recorded

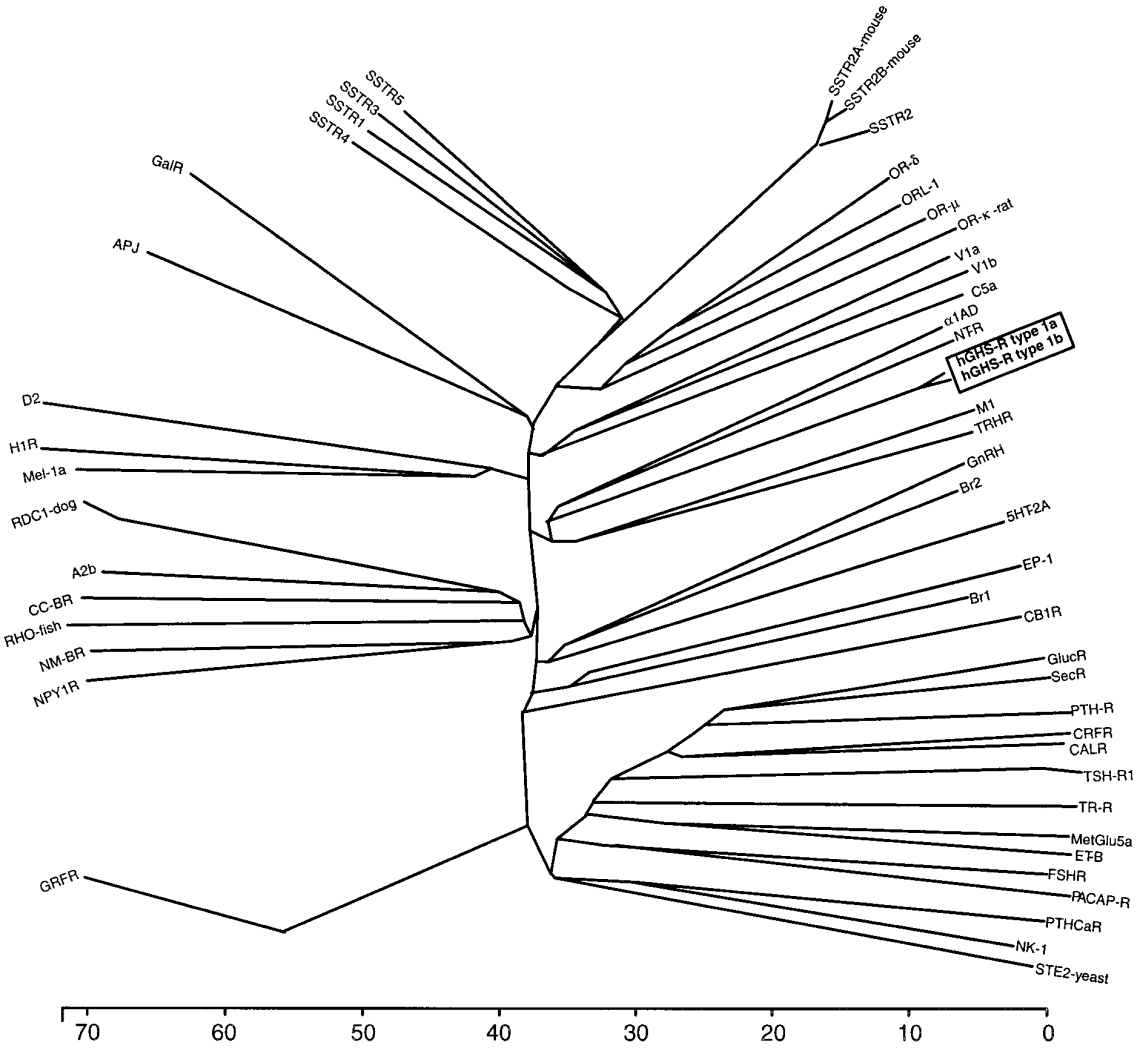

FIG. 12. Dendrogram of GHS-R and other G protein-coupled receptors. Database searches (Genbank 92, EMBL 43, Swiss-Prot 31, PIR 45, dEST (Gbest 92), Prosite 12), sequence alignments, and analysis of the GHS-R nucleotide and protein sequences were carried out using the GCG Sequence Analysis Software Package (Madison, WI; pileup, peptide structure and motif programs), FASTA and BLAST search programs, the PC/Gene software suite from Intelligenetics (San Francisco, CA; protein analysis programs) and Lasergene software (DNA Star, Madison, WI). The amino acid sequence of representative members (51 sequences) for all known classes (Families I-IV and pheromone) of G protein-coupled receptors were used to construct the dendrogram using the clustal method (PAM-250; gap and length penalty = 10). The length of each pair of branches represents the distance between sequence pairs. The scale *below* the tree measures the distance between sequences. Units indicate the percent of substitution events. All receptor sequences aligned to human GHS-R type Ia and type 1b cDNAs were human unless otherwise noted, and accession numbers are for the SwissProt database, GenBank database (designated with "G"), or PIR database ("P"): 5HT-2A, serotonin, P28223; RDC-1, orphan receptor, A39714; somatostatinR4, somatostatin, A47457; V1a, vasopressin, A53046; V1b, vasopressin, A55089; A2b, adenosine, P29275; M1, muscarinic; P11229; APJ, orphan, P11229; C5a, chemotactic, P21730; CASR, extracellular calcium-sensing; P41180; CB1, cannabinoid, P21554; CRF, corticotropin-releasing factor, P34998; FSHR, FSH, P23945; CC-BR, gastrin/cholecystokinin type B, P32239; GRFR, GH-releasing, Q02643; GnRH, gonadotropin-releasing, P30968; CALR, calcitonin, X69920 (G); GlucR, glucagon, U03469 (G); OR-δ, opioid, U10504 (G); Mel-1a, melatonin, U14108 (G); SecR, secretin, U20178 (G); PTHCaR, parathyroid cell calcium-sensing, U20759 (G); Br1, bradykinin, U22346 (G); α1AD, alpha-1 adrenergic, L31772 (G); GalR, galanin, L34339 (G); H1R, histamine, D14436 (G); TRHR, TRH, D16845 (G); MetGlu5a, metabotropic glutamate, JC2132 (P); α1AD, alpha-1 adrenergic, JC2331 (P); Br2, bradykinin, JH0712 (P); NK-1, substance P, P25103; NM-BR, neuromedin-B, P28336; NT-R, neurotensin, P30989; NPY1R, neuropeptide Y, P25929; OR-μ, opioid, P35372; RHO-fish, rhodopsin, P35356; EP-1, prostaglandin E1, P34995; PTH-R, PTH, Q03431; OR-κ, opioid-rat, S39015 (P); ORL-1, opioid-orphanin FQ, S43087 (P); ET-B, endothelin, S44866 (G); D2, dopamine, S62137 (G); STE2-yeast, pheromone α factor, P06842; SSTR2, SSTR3, SSTR5, SSTR2A-mouse, SSTR2B, SSTR1, somatostatin, P30874, P32745, P35346, P30875, P30934, P30872, respectively; PACAP-R, pituitary adenylate cyclase-activating peptide, D17516 (G); TR-R, thrombin, P25116; TSH-R1, TSH, S49816 (G).

within 5–15 min after dosing, and L-692,585, consistent with its *in vitro* potency, was effective at approximately 1/20th of the dose compared with L-692,429 (111). No sex-related difference was detected and, apart from small transient increases in ACTH and cortisol, both compounds were very selective at stimulating GH release (89, 103). In a more extensive chronic study in which L-692,585 was given once daily for up to 14 days, desensitization to repeated dosing was not observed. Increases in IGF-I were evident 6 h after dosing, but the increase was transient and IGF-I levels re-

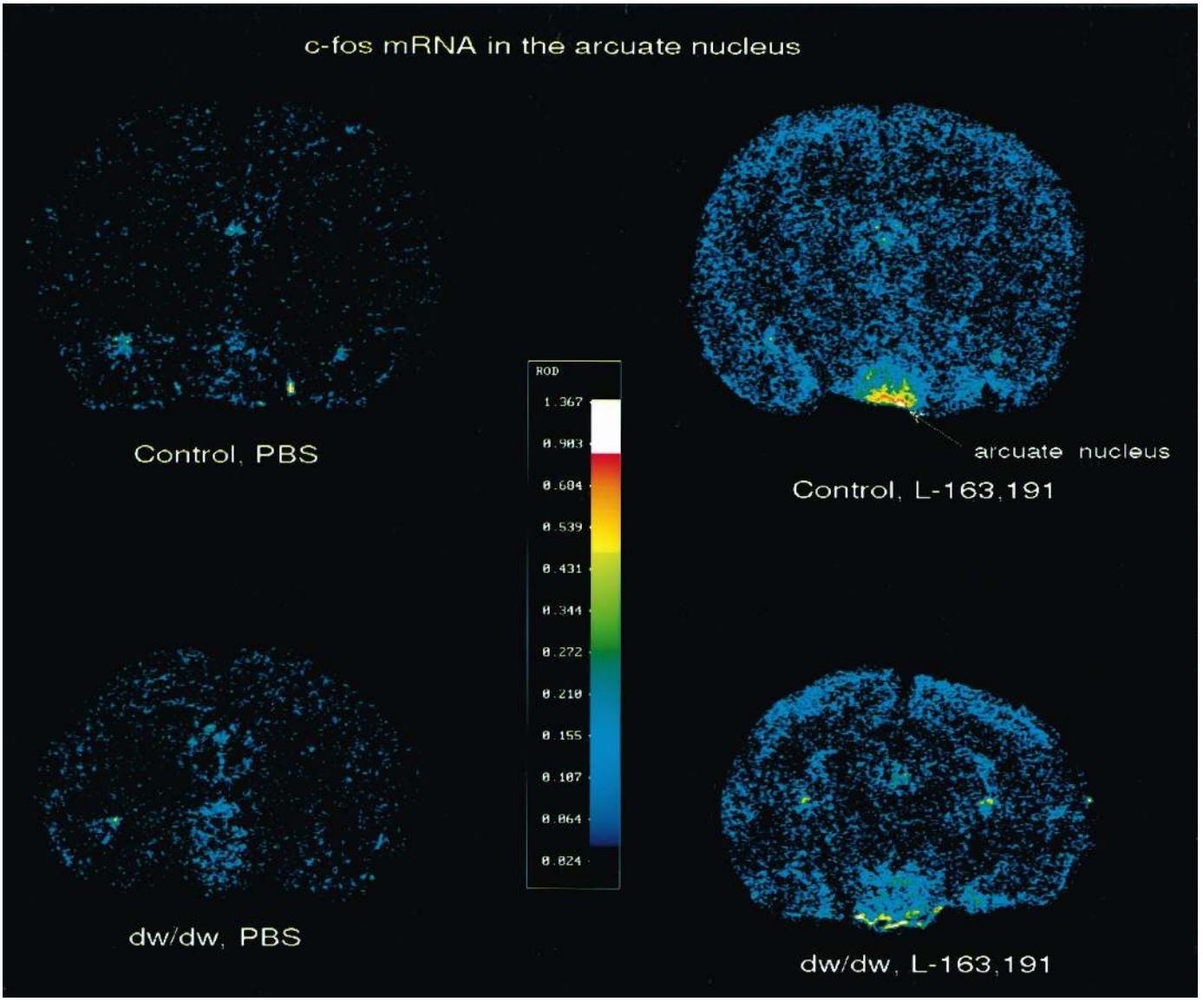

FIG. 13. Activation of c-*fos* gene expression in the central nervous system of control and *dw/dw* mice after intraperitoneal MK-0677 administration (89). Wild type C57 BL/6J mice and *dw/dw* mice were injected intraperitoneally with either PBS or MK-0677 (50 mg/kg) and killed 40 min later. *In situ* hybridizations for c-*fos* mRNA were performed on coronal brain sections. Images have been artificially colored: *blue* represents background; *white* e represents the most intense signals (23).

turned to baseline within 24 h. PRL, insulin, and T_4 levels were unaltered over the course of the study (111).

Pharmacokinetic oral bioavailability measurements had established that MK-0677 was a viable candidate for once-daily oral dosing (50, 52). To determine whether the effects on GH release could be sustained during repeated oral treatment, dogs were treated with MK-0677 (1 mg/kg) for 4 days. On days 1 and 4, blood was collected at 15-min intervals for 8 h and assayed for GH. MK-0677 treatment resulted in sustained amplification of the pulsatile profile of GH. However, because the magnitude of GH release caused by MK-0677 decreased markedly by the fourth day (59), a second study of 14 days duration was implemented to determine whether complete desensitization would occur during more prolonged treatment. Although reduced amplitude of the GH response to MK-0677 was again observed by day 4, treatment for up to 14 days did not result in further attenuation. A sustained increase in serum IGF-I levels accompanied the reduced amplitude of GH release (54). Interestingly, a similar study with the shorter acting secretagogue L-692,585 gave neither a reduced GH response nor a sustained increase in IGF-I levels during repeated daily treatment (111). When dogs were dosed with MK-0677 on alternate days for up to 9 days, reduced GH responses to repeated dosing was not evident. This particular dosing regimen allowed IGF-I to return to basal levels before dogs received the next dose of MK-0677. Based on these observations it was speculated that tachyphylaxis might be associated with increases in IGF-I rather than desensitization of the GHS-R. To address this possibility, dogs were treated with MK-0677 on day 1; on days 2 and 3 the dogs were dosed with porcine GH; and on day 4 the dogs were treated with

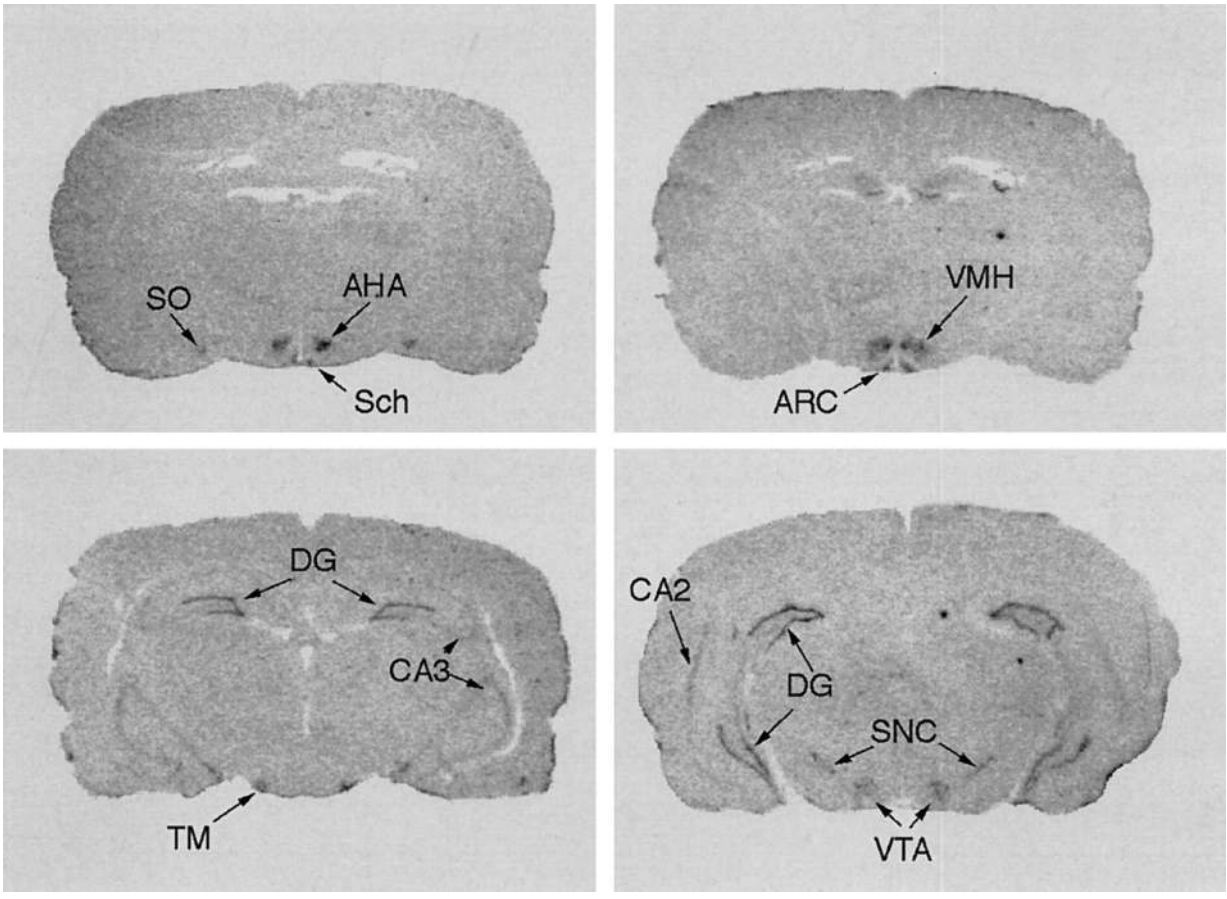

FIG. 14. *In situ* hybridization of GHS-R mRNA in rat brain (105). ^{33}P-Labeled rat GHS-R oligonucleotide probes were used for *in situ* hybridization of coronal rat brain sections. Signals were highly specific and could be displaced by 100-fold molar excess of cold probe. Abbreviations are as follows. *Top left panel*: AHA, anterior hypothalamic area; Sch, suprachiasmatic nucleus; SO, supraoptic nucleus. *Top right panel*: ARC, arcuate nucleus; VMH, ventromedial hypothalamus. *Bottom left panel*: DG, dentate gyrus; CA2/CA3, CA2 and CA3 regions; TM, tuberomammillary nucleus. *Bottom right panel*: SNC, pars compacta of substantia nigra; VTA, ventral tegmental area. [Reprinted from X.-M. Guan *et al.*: *Mol Brain Res* 48:23–29, 1997, with kind permission from Elsevier Science-NL, Sara Burgerhartstraat 25, 1055 KV Amsterdam, The Netherlands.]

MK-0677. At the time of treatment on day 4, GH concentrations had returned to basal levels but IGF-I was still elevated. The GH response to MK-0677 on day 4 was markedly attenuated compared with day 1. Thus reduced responsiveness to chronic MK-0677 treatment is not necessarily due to desensitization of the GHS-R but might be explained by reduced responsiveness of the GH/GHRH axis caused by a sustained increase in IGF-I (54). Perhaps chronically, IGF-I feeds back on arcuate neurons to reduce the expression of both GHRH and the GHS-R. The demonstration that the magnitude of the stimulatory effect of MK-0677 on GH release is limited by negative feedback is very attractive clinically because it prevents hyperstimulation of the GH/IGF-I axis.

Like GHRP-6, L-692,429 and L-692,585 also cause transient increases in ACTH and cortisol (110, 111). The effect on ACTH is probably mediated by action of the secretagogues on the hypothalamus, because in cultured pituitary cells the peptidomimetics do not significantly increase ACTH release. MK-0677 also increased cortisol levels when given acutely; however, during chronic administration the stimulatory effect on cortisol became insignificant (53, 54). When MK-0677 was given chronically to dogs on alternate days, just as observed with GH, the cortisol response was not attenuated (54). These results suggest that the GH and cortisol responses are linked and perhaps similarly attenuated during repeated daily treatment through an IGF-I-mediated pathway.

Interesting anabolic activities were reported for the Genentech compound G-7039. Body weight gain in 150-day-old female Sprague Dawley rats was significantly greater when G-7039 was administered by subcutaneous minipump twice daily for 14 days as compared with the body weight gain after subcutaneous minipump infusion of G-7039 for 14 days (58). The implication is that optimal growth requires intermittent administration of these secretagogues rather than continuous treatment. It remains to be tested whether this applies to species other than rats.

B. Clinical studies in humans

GH treatment may prove to be beneficial to a variety of subjects other than GH-deficient children. Most individuals over the age of 60 yr might be considered GH deficient according to young adult standards (112–114), and it has

been suggested that this relative deficiency is responsible for decreases in bone and muscle mass and increased adiposity during aging (115). Since GH increases bone turnover, GH in combination with an inhibitor of bone resorption may have great benefit in severe osteoporosis. GH deficiency is associated with lipid profiles that favor the likelihood of atherosclerosis and mortality due to cardiovascular disease (116–119). Depressed subjects present a significant decrease in 24-h GH production (120). Whether the decreased amplitude of the GH pulses reported in major depressive illness is due to changes in the activity of neurotransmitters or is related to GH itself remains to be established.

Perhaps the greatest potential for GH replacement is in the frail elderly population. Although pulsatile GH secretion declines during aging, rodent studies show that the pituitary gland continues to synthesize GH and remains responsive to GHRH (121, 122). The advantage of increasing GH in the elderly is suggested by the recent demonstration that in a population of healthy men over 60 yr old, once-daily GH treatment over a 3-month period increased lean body mass, muscle mass, and thigh strength measured by isokinetic dynamometry (123). Therefore, identification of a compound that rejuvenates the GH/IGF-I axis provides a way to optimally evaluate the potential clinical benefits of reversing GH deficiency in the musculoskeletally impaired elderly (59, 124). The GHRPs and the peptidomimetic GH secretagogues provide a more physiological approach to GH replacement; however, based on our understanding of the mechanisms involved, subjects must have an intact hypothalamic/pituitary axis and produce normal GH. Consequently, in certain instances, treatment with recombinant GH is the only option.

1. L-692,429. A series of studies demonstrated that the GHRPs showed reproducible GH-releasing activity in humans (18, 125–132). The first nonpeptide GH secretagogue discovered, L-692,429, was shown to be a mimetic of GHRP-6 (29). Based on small animal studies, L-692,429 had improved oral bioavailability (4%) compared with the GHRPs. Although its bioavailability was considered inadequate for a clinical development candidate, L-692,429 was selected to test the concept that this new class of compounds would stimulate GH release in humans. Its efficacy, tolerability, and selectivity in clinically relevant target groups were determined. Healthy normal volunteers and the elderly tolerated L-692,429 well. Dose-dependent increases in GH were observed that were accompanied by small, but significant, transient increases in cortisol (133, 134). Elderly subjects were somewhat less responsive than young adults as were subjects chronically treated with glucocorticoids (134, 135). However, this deficit was overcome by administering a 3-fold higher dose. L-692,429 also stimulated GH release in obese subjects (136).

Constant infusion of L-692,429 in healthy elderly adults enhanced pulsatile GH secretion similar to that observed with GHRP-6 (128, 137). Infusion of 0.05 -0.1 mg/kg·h for 12–24 h increased pulsatile GH concentrations in older adults (60–80 yr old) in a dose-dependent manner (137). The number of GH-secretory pulses and the half-life of GH disappearance were not markedly affected by L-692,429 infusion; rather, the pulse amplitude was increased. Remarkably, the profile and GH levels resulting from L-692,429 infusion into the elderly approximated that of young adults (137). These results indicated that the peptidomimetic GH secretagogues had excellent potential for treatment of GH deficiency in humans. Since constant infusion provided amplification of a physiological profile, a molecule having high oral bioavailability and appropriate half-life for once-daily treatment was sought.

2. MK-0677. Based on studies in rodents and beagle dogs, MK-0677 was identified as having appropriate properties for a once-daily oral drug capable of sustaining amplification of pulsatile GH release (54, 59). Studies in humans showed MK-0677 to be well tolerated, and once-daily oral administration was sufficient to increase serum concentrations of both GH and IGF-I. Seventeen male and 15 female healthy elderly volunteers (64–81 yr old) were treated orally once daily with MK-0677 at doses of 2, 10, and 25 mg in a randomized, double-blind, placebo-controlled trial. Serum was collected every 20 min for 24 h at baseline and after each of two 14-day treatment periods the serum was analyzed for GH, cortisol, and PRL. The assessment of effects on pulsatile GH release was evaluated by three independent algorithms. MK-0677 increased GH concentrations dose dependently (138, 139). Serum IGF-I concentrations were also significantly increased at both the 10- and 25-mg doses while the 2-mg dose was ineffective. At the 25-mg dose the mean 24-h GH concentration increased by 97 ± 23% due to enhancement of preexisting pulsatile GH secretion. Similarly, in these elderly subjects, serum IGF-I levels increased from 141 ± 21 μg/liter into the normal range for young adults (219 ± 21 μg/liter). These increases were sustained for at least 28 days (265 ± 29 μg/liter). Most importantly, serum cortisol, diurnal cortisol pattern, and urinary free cortisol remained in the normal range and were all unchanged compared with the placebo group (138, 139). Also, thyroid hormones were not significantly affected by MK-0677. Small increases in PRL from pretreatment levels were observed, but the concentrations remained in the normal range. After 4 weeks, MK-0677 treatment was associated with a significant increase in fasting glucose (5.4 ± 0.3 to 6.8 ± 0.4 mmol/liter) and insulin concentrations. The increases in glucose were correlated with body mass index, suggesting that impaired glucose tolerance might be an issue in subjects with predisposing risk factors. However, it is likely that insulin sensitivity would improve over time coincident with anticipated improvements in body composition. Consistent with a GH increase, IGF-II and IGF binding protein-3 (IGFBP-3) increased, and IGFBP-1 and IGFBP-2 levels decreased. Figure 15A illustrates the sustained increases in pulsatile GH release observed in elderly men and women during repeated daily dosing with MK-0677. The GH and IGF-I levels attained are similar to those of healthy young adults. Cortisol levels (Fig. 15B) are unchanged, demonstrating the selectivity of this peptidomimetic GH secretagogue (139).

IX. Regulation of Pulsatile GH Release

A. GHRH and somatostatin

GH secretion is markedly pulsatile in all species studied (140–144). The biological significance of episodic secretion

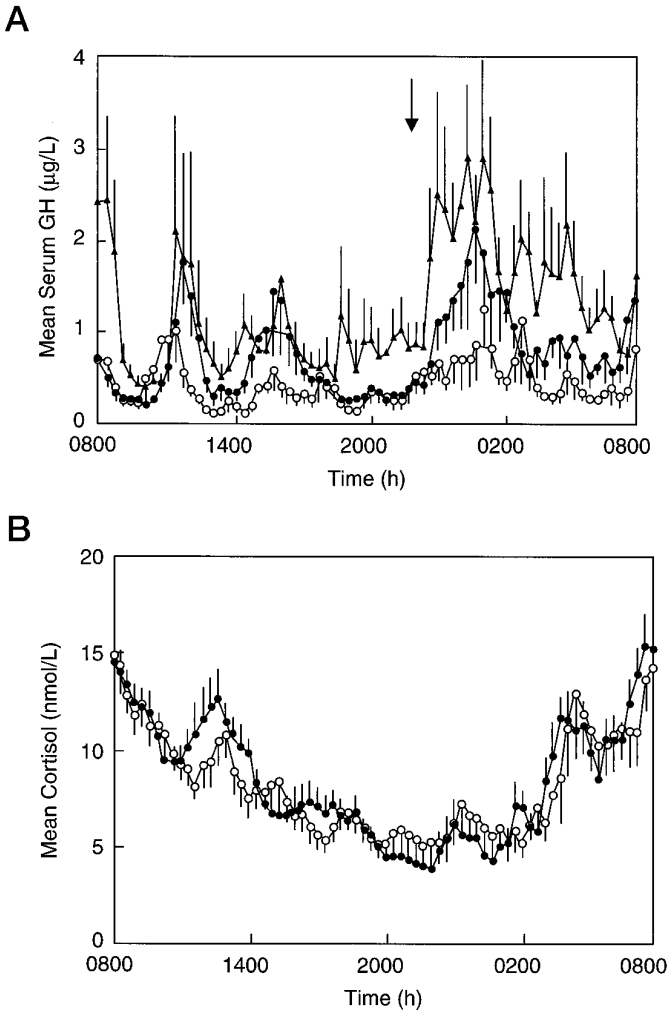

FIG. 15. A, Rejuvenation of the GH axis in elderly men and women treated for 14 days once daily with MK-0677 showing a clear increase in amplitude of pulsatile GH release. Mean (±SE) serum GH concentrations after 2 weeks of treatment with placebo (○; n = 10), 10 mg/day MK-0677 (●; $n = 12$) and 25 mg/day MK-0677 (▲; $n = 10$). Evening treatment time (between 2200 and 2300 h) is indicated by the *arrow*. B, Effect of oral 25 mg/day MK-0677 administered to 10 elderly men and women once daily for 14 days on serum cortisol. Serum cortisol at baseline (○) and on day 14 (●). Blood samples were collected every 20 min. [Reproduced with permission from I. M. Chapman *et al.*: *J Clin Endocrinol Metab* 81:4249–4257, 1996 (139). © The Endocrine Society.]

has been illustrated in GH-deficient animals in which GH replacement is more efficient in improving growth when given in a pulsatile pattern rather than by constant infusion (145). Before describing how we believe the peptidomimetic GH secretagogues themselves are involved in regulating pulsatility, we should first review what is currently understood about the control of pulsatile GH release.

The experimental evidence points to GH periodicity being self-entraining. For example, when GH is given exogenously at intervals of 3 h, approximately in phase with endogenous GH pulses, the exogenous and endogenous GH peaks become entrained (146). If the exogenous pulses are repeated more frequently (every 90 min), the regular endogenous pulsatility disappears (146). Entrainment of the 3-h pulses can be accomplished also by administering a GHRH analog (147) or L-692,585 instead of GH at 3-h intervals (7, 87). These regular episodic GH peaks at 3- to 3.5-h intervals are best explained by GH-induced feedback rather than by changes in environmental factors or ultradian rhythms, because in male rats, irrespective of light-dark cycle, the time between GH peaks is unaffected (148). GH does not inhibit GH release from primary cultures of rat pituitary cells (149), and introduction of GH into the third ventricle inhibits endogenous GH release (150); therefore, GH-negative feedback is probably mediated at the hypothalamic level, perhaps by retrograde transport through the portal vessels (151, 152). Persuasive evidence for hypothalamic GH-mediated negative feedback has been elegantly illustrated by showing that increased pulsatility follows administration of antisense GH receptor mRNA into the brain (153).

Pulsatile GH release appears to be related to reduced GHRH secretion and increased somatostatin secretion during GH troughs (154–158). Experiments using GHRH antibodies and GHRH antagonists show that GH pulses are dependent on the release of GHRH (159, 160). Hypothalamic/pituitary stalk disconnection results in loss of normal GH pulsatility in sheep (161), suggesting that pulsatility is controlled centrally by changes in GHRH, somatostatin, or some other hypothalamic factor(s). When GHRH is constantly infused into humans, pulsatile GH release is amplified and sustained. Thus, changes in GHRH alone do not necessarily explain GH pulsatility (128, 137, 162) and implicate an important role for somatostatin. Increases in somatostatin suppress endogenous GH release (146, 163, 164), whereas a decline in somatostatin facilitates the release of GHRH from the hypothalamus and GH release from somatotrophs (165, 166).

The strict adherence to a 3-h cycle of GH pulsatility is likely governed by the pharmacodynamics of GH on somatostatin tone. The rationalization that desensitization to somatostatin explains the 3-h cycle is untenable because continuous infusion of a somatostatin agonist, MK-678, prevents pulsatile GH secretion for at least 12 h (K. Cheng and R. G. Smith, unpublished observations). Studies demonstrating that endogenous GH pulsatility is sustained when somatostatin is neutralized by infusion of somatostatin antibodies (154) is not inconsistent with the notion of a central role for somatostatin since these antibodies may not be capable of readily accessing and thereby influencing the appropriate somatostatin neurons. Whether GH feeds back directly on somatostatin-containing neurons or whether the effect is indirectly mediated by release of another mediator, such as NPY, from arcuate neurons is currently being elucidated (167).

An important question is whether pulsatility is also controlled at the level of the pituitary gland. If so, by monitoring hypothalamic secretions, it should be possible to correlate peaks in GHRH and troughs in somatostatin with GH peaks in peripheral blood. When GHRH and somatostatin were monitored in the hypothalamic-pituitary portal vessels of conscious sheep and pigs to test this possibility, the results were far from satisfying. In one study the occurrence of a simultaneous GH and GHRH peak associated with a fall in somatostatin occurred 48% of the time. A GH peak in the

absence of a change in GHRH but accompanied by a fall in somatostatin was observed 18.5% of the time (168). Another study gave a 62% match of GH and GHRH peaks ($P < 0.02$) without a significant correlation with somatostatin troughs (169). Recently, using a transorbital approach, Drisko *et al.* (170) measured somatostatin and GHRH in the portal vessels of pigs and observed a closer correlation of GH peaks with somatostatin troughs (63%) than with GHRH peaks (33%) (J. E. Drisko and G. J. Hickey, unpublished observations). The experimental findings are difficult to interpret conclusively for various reasons. Limited sensitivities of the GHRH and somatostatin assays do not allow quantification at 0.5–1 min sampling intervals. Ideally, such frequent sampling is important because perifusion experiments show that pituitary cells respond to somatostatin and GHRH within 1 min (27). The problem is exacerbated because the half-lives of these hypothalamic hormones are relatively short. Furthermore, the concentrations of GHRH and somatostatin in portal blood are very low, and based on *in vitro* generated GHRH and somatostatin dose-response curves for modulating GH release, small changes in GHRH and somatostatin concentrations are likely to cause more profound increases in GH. Therefore, because small dynamic changes in somatostatin and GHRH are difficult to accurately and reproducibly quantitate in portal blood, the precise roles of these hypothalamic hormones in controlling GH pulsatility at the level of the pituitary gland remain obscure.

B. The role of GHS-R

The sites of action of the peptidomimetic GH secretagogues are illustrated in Fig. 16. GHS-Rs have been identified in the pituitary and hypothalamus, and modification of electrical activity of arcuate neurons has been demonstrated. Since ligands for the GHS-R behave as functional antagonists of somatostatin and amplifiers of GHRH activity, it is predictable that the GHS-R ligands would amplify GH secretion *in vivo.* Less obvious is how they sustain GH pulsatility. Although sustained exposure to MK-0677, L-692,429, L-692,585, and GHRP-6 amplifies pulsatile GH release for at least 24 h (59, 87, 128, 137, 139), when pituitary cells are exposed to these secretagogues *in vitro* the cells become refractory to repeated stimulation within minutes (27, 171). The paradox between the *in vivo* and *in vitro* results must be explained by other factors that modulate GHS-R function *in vivo*.

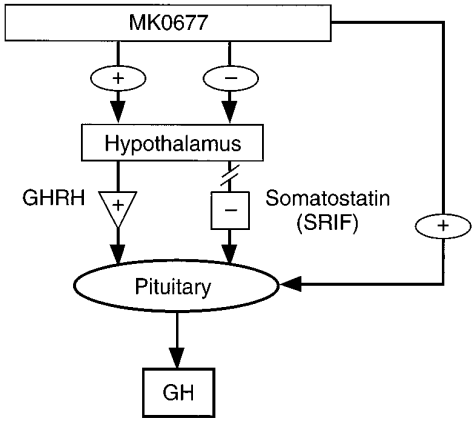

FIG. 16. Sites of action of the peptidomimetic GH secretagogues. The ligands for the GHS-R act directly on somatotrophs to cause GH secretion, amplify the affects of GHRH, and behave as functional antagonists of somatostatin. They also act in the arcuate nucleus to increase the activity of GHRH-containing neurons.

The optimal effects of GHS-R ligands on GH release are only observed when the hypothalamic pituitary axis is intact, suggesting an essential permissive role for GHRH (172–174). In hypothalamic-pituitary stalk transected animals, the GH response to a peptidomimetic GH secretagogue is very weak but becomes equivalent to that of control animals when GHRH is coadministered (172). In intact animals administration of GHRH antibodies markedly inhibits the GH response to the GHRP, hexarelin (175). GHRH, like the GHS-R ligands, causes desensitization of pituitary cells during prolonged infusion *in vitro* (27) and when continuously infused *in vivo* produces episodic GH release (162). These results suggest that changes in GH and somatostatin are sufficient to control GH pulsatility *in vivo* when hypothalamic control of GHRH secretion is bypassed. To test the role of somatostatin we used a pituitary cell perifusion system (27) and superimposed pulses of somatostatin upon a constant perifusion of GHRH and GHRP-6. As anticipated, we achieved a pulsatile profile of GH release from the pituitary cells. Surprisingly, after the somatostatin pulse a rebound effect caused more GH to be released (A. D. Blake and R. G. Smith, unpublished results) suggesting that somatostatin is capable of preventing desensitization to the GHS-R ligands. Subsequent studies showed that, in a dose-dependent manner, somatostatin is capable of preventing tachyphylaxis of pituitary cells to GHRH, GHRP-6, and the peptidomimetic GH secretagogues (K. Cheng and R. G. Smith, unpublished observations). These *in vitro* experiments demonstrate that somatostatin, in addition to its well known role in controlling GH release from somatotrophs, also plays an important role in controlling the sensitivity of the somatotroph to GHRH and the GHS-R ligand. Based on these observations, a mathematical model was derived that supported the notion that pulsatility can be explained solely by changes in somatostatin concentrations (176). These results are consistent with suggestions that oscillations in somatostatin are involved in maintaining GH pulsatility *in vivo* (153–158, 162, 165, 177).

The *in vitro* studies described above suggest that, pharmacologically, GH pulsatility can be controlled at the level of the pituitary. However, there is considerable evidence that, physiologically, regulation is exerted at the level of GHRH neurons in the arcuate nucleus. Electrophysiology and c-*fos* activation studies on hypothalamic neurons (86, 88, 97) suggest that ligands for the GHS-R regulate pulsatility, at least in part, by inducing release of GHRH from arcuate neurons. Consistent with this prediction, in conscious sheep, GHRPs have been shown to increase GHRH and the frequency of GHRH pulsing in the hypothalamic-portal vessels without affecting somatostatin levels (98, 99). However, experiments with the peptidomimetics have given inconsistent results. Although we consistently observed elevations in GH in peripheral blood after treatment of sheep with L-692,585, only on rare occasions were we able to measure significant increases in GHRH in portal blood (59). Similar frustrating

results were obtained in pigs and rhesus monkeys (J. E. Drisko, G. J. Hickey, H. G. Spies, and R. G. Smith, unpublished results). Although these findings might indicate the peptidomimetics act solely on the anterior pituitary gland, this conclusion does not explain why L-692,585 causes GH release and activates arcuate neurons at lower doses when injected into the third ventricle compared with the intravenous route (7, 86, 87). The lack of a perfect correlation between acute GHRH and GH release in response to L-692,585 might simply be explained by variability among animals in transporting L-692,585 across the blood-brain barrier. Clearly, rapid brain penetration is essential to synchronize hypothalamic and pituitary events. Indeed, after intravenous injection of L-692,585, recordings from electrodes placed on arcuate neurons of rats showed a 5- to 10-min lag period before excitation was detected (86, 88, 90). Therefore, while stimulation of GHRH release by the peptidomimetics has not been causally linked to increased GH secretion, reproducible, more intensive investigations are needed before a relationship can be ruled out.

Bowers (4) has argued that the increased activity of the GHS-R ligands *in vivo* compared with *in vitro* cannot simply be explained by stimulation of GHRH release and inhibition of somatostatin tone. These conclusions are based on observations that in the presence of maximum dosages of GHRH, or when animals were pretreated with somatostatin antibodies, synergism in response to GHRPs was still observed. Additional supporting evidence for Bowers' view is that the magnitude of the increases in cAMP measured in rat pituitary gland after administration of GHRP and GHRH did not correlate with the level of GH release (4). With GHRP, the increase in cAMP was low and the increase in GH release was high, whereas GHRH provided a large increase in both cAMP and GH. Since cAMP increases GH secretion, if GHRP caused GHRH release, a closer correlation would have been anticipated. Another study revealed the concentrations of GHRP, GHRH, and GHRP + GHRH *in vitro* and *in vivo* that inhibited the activity of somatostatin on GH release by 50% (4). While the effects of GHRP and GHRH were found to attenuate the effects of somatostatin equally *in vitro* and *in vivo*, the combined effects of GHRP + GHRH were additive *in vitro* but synergistic *in vivo*. Bowers concluded from these studies that GHRPs cause the release of an unknown hypothalamic factor (U-factor) that results in a synergistic effect on GH release (4). However, administration of GHRPs and somatostatin exogenously to animals is complicated by how well these compounds penetrate the brain and activate specific hypothalamic neurons. Furthermore, the magnitude of GH release regulated by GHS-R ligands, GHRH, somatostatin, and cAMP is not a quantitative linear relationship. Hence, while Bowers' hypothesis is very appealing, the need for a U-factor to explain the synergistic effects of the GHS-R ligands awaits confirmation.

Our working model is that, physiologically, GH self-entrains its pulsatility through the coupling of three biological oscillators: somatostatin, GHRH, and the natural ligand for the GHS-R. However, when a GHS-R ligand is administered exogenously, it acts dominantly to antagonize somatostatin and stimulate GHRH release. This results in synergy between GHRH and the GHS-R ligand to cause increased GH release from somatotrophs. GH then feeds back on the hypothalamus to entrain a new cycle by increasing somatostatin tone on GHRH-containing neurons, thus inhibiting GHRH and GH release. Hence, administration of a GHS-R ligand is capable of resetting the coupled oscillators.

It is tempting to speculate on the mechanism of the interplay between somatostatin and the GHS-R. As discussed above, somatostatin appears to prevent desensitization of the GHS-R. However, until somatostatin falls to a critical concentration, the GHS-R ligand cannot overcome the inhibitory effects of somatostatin on GH release because somatostatin receptors are present in excess over GHS-R (60). The inhibitory effects of somatostatin on GH release and maintenance of GHS-R in an active state probably occurs through key phosphorylation/dephosphorylation reactions since somatostatin causes translocation of a tyrosine phosphatase and activation of protein phosphatase-2A (178–181). MK-0677 activates protein kinase C, perhaps causing tyrosine phosphorylation of a K^+ channel, as described for the m1 muscarinic receptor (182). This would explain the observed depolarization of the somatotroph membrane and stimulation of GH release. The phosphorylated K^+ channel is a potential substrate for the tyrosine phosphatase activated by somatostatin. Dephosphorylation of the channel would cause hyperpolarization and inhibit GH release. The serine/threonine phosphorylation sites on the GHS-R are likely involved in receptor desensitization, and we speculate that somatostatin-induced protein phosphatase-2A regulates the phosphorylated state of phosphorylated GHS-R. In spite of the fact that a proportion of phosphorylated GHS-Rs may be reactivated by somatostatin, GH release will still be tightly controlled because the somatotrophs remain hyperpolarized until somatostatin tone is reduced (16). Thus, GH could self-entrain its pulsatility indirectly through a somatostatin feedback loop that acts in a concentration-dependent manner on the GHS-R and K^+ channels.

X. Concluding Comments

In summary, a class of orally active small molecules have been described that are mimetics of the synthetic peptide GH secretagogue GHRP-6. The receptor for these molecules has been cloned and shown to be distinct from that of the GHRH receptor. This new receptor is G protein coupled and does not obviously fall into a known family of G protein-coupled receptors. However, the GHS-R is highly conserved across species, suggesting that an unidentified hormone for this receptor plays an important physiological role in the control of pulsatile GH release. The importance of the GHS-R pathway might be exemplified in GH-deficient children because the majority of children respond to ligands that bind to the GHS-R (125). Perhaps these children lack the natural ligand for the receptor. If this speculation is true, individuals lacking the GHS-R or its natural ligand would have growth-retarded phenotypes because of reduced amplitude of episodic GH release. Similarly, the attenuation of GH pulsatility during aging, which can be corrected by administration of MK-0677, is suggestive of reduced production of the endogenous ligand. Based on the localization of GHS-R expression in the

brain, a decline in the ligand concentration might be associated with age-related deficits in cognitive function. Indeed, expression of the GHS-R in specific regions of the brain not implicated in GH release implies a much broader role for the natural ligand in human physiology.

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