A Receptor in Pituitary and Hypothalamus That Functions in Growth Hormone Release

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Small synthetic molecules termed growth hormone secretagogues (GHSs) act on the pituitary gland and the hypothalamus to stimulate and amplify pulsatile growth hormone (GH) release. A heterotrimeric GTP-binding protein (G protein)–coupled receptor (GPC-R) of the pituitary and arcuate ventro-medial and infundibular hypothalamus of swine and humans was cloned and was shown to be the target of the GHSs. On the basis of its pharmacological and molecular characterization, this GPC-R defines a neuroendocrine pathway for the control of pulsatile GH release and supports the notion that the GHSs mimic an undiscovered hormone.

We sought to correct GH deficiencies by identifying a compound capable of mimicking or amplifying the biological oscillator that regulates GH release. GH secretion is controlled by at least two hypothalamic hormones; growth hormone–releasing hormone (GHRH) stimulates GH release from somatotrophs in the anterior pituitary gland, and somatostatin inhibits GH release (*1*). A synthetic hexapeptide (GHRP-6) that contains D–amino acids stimulates GH release by a pathway distinct from that of GHRH, which implies the presence of a third receptor (*2, 3*). Nonpeptide mimetics of GHRP-6 with improved pharmacokinetics and oral bioavailability (L-692,429; MK-0677) also exist (*4*) and suggest that this class of GHSs mimics a natural hormone involved in episodic GH release. These synthetic molecules have potential clinical benefit to humans (*5, 6*). To pursue this hypothesis, we sought a receptor selective for the GHSs.

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GHSs cause depolarization and inhibition of potassium channels (*7*), induce a transient increase in the concentration of intracellular calcium in somatotrophs (*8, 9*), increase intracellular concentrations of inositol trisphosphate (*10, 11*), and cause an associated increase in activity of protein kinase-C (*11*). The biochemical basis for these changes indicated that a GPC-R may be activated. A high-affinity, low abundance binding site for ^{35}S-labeled MK-0677 exists in the anterior pituitary gland and hypothalamus (*12*). The binding is Mg^{2+}-dependent and is inhibited by stable guanosine triphosphate (GTP) analogs, further supporting the notion that the GHS receptor (GHS-R) is G protein–coupled.

Xenopus oocytes injected with polyadenylated [poly(A)$^+$] RNA from swine pituitary exhibited oscillatory increases in calcium-activated chloride currents induced by MK-0677 (10 to 100 nM) or GHRP-6 (1 μM), which indicated expression of the GHS-R (Fig. 1A). The responses were generally small (86 ± 32 nA, n = 32) and were infrequently observed (positive results with oocytes from only 6 of 18 frogs), even though responses with other ligands, thyrotropin-releasing hormone (TRH), and gonadotropin-releasing hormone (GnRH) were more consistently measured. Rat pituitary poly(A)$^+$ RNA failed to give a response under these conditions.

We improved the sensitivity of the assay by co-injecting synthetic complementary RNA (cRNA) that encodes the bioluminescent Ca^{2+}-sensitive protein aequorin and cRNA for the G protein α subunit $G\alpha_{11}$ (Fig. 1B) (*13, 14*). Other Gα subunits ($G\alpha_q$, $G\alpha_{16}$, $G\alpha_o$, $G\alpha_{13}$, $G\alpha_{i1}$, $G\alpha_{i3}$, and $G\alpha_s$) given singly or in combination, failed to revive MK-0677 responsiveness (*15*). The pharmacology of the swine GHS-R was investigated with several GHSs (*15*). Bioluminescent responses were obtained with 1 nM MK-0677. The biologically active benzolactam L-692,429 also evoked responses, whereas its biologically inactive enantiomer L-692,428 (*4*) did not. Human pituitary, rat pituitary, and rat hypothalamus poly(A)$^+$ RNA also gave positive responses to MK-0677 when co-injected with $G\alpha_{11}$ cRNA, whereas poly(A)$^+$ RNA from control tissues (including rat cerebral cortex, rat brain, and human fetal liver) did not (Fig. 1C). This indicated that a GHS-R, most likely coupled to $G\alpha_{11}$, is present in human pituitary gland, which may explain the re-

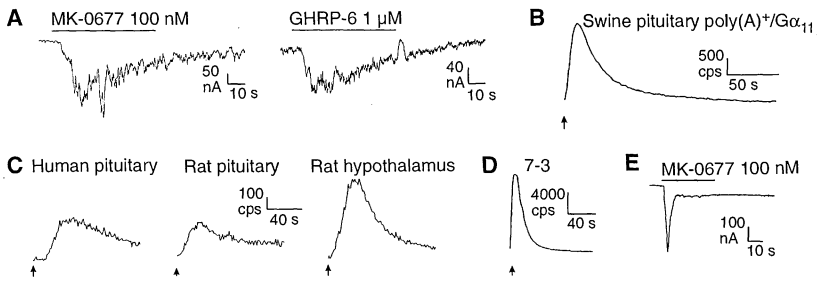
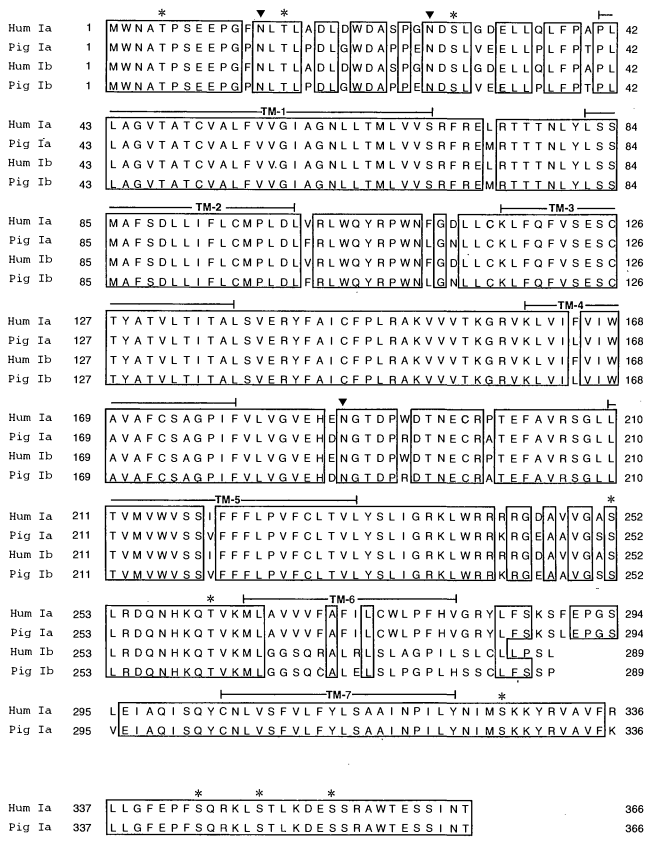


Fig. 1. Expression of the swine type Ia GHS-R in *Xenopus* oocytes. (**A**) Oocytes were injected with poly(A)$^+$ RNA (50 ng) from swine pituitaries, and membrane currents were recorded at −80 mV. Current traces for 100 nM MK-0677 (left) and 1 μM GHRP-6 (right) are shown. The horizontal line indicates the duration of ligand exposure. (**B** and **C**) Aequorin bioluminescence responses with G protein addition [2 ng; ($G\alpha_{11}$ cRNA at a 1:9 ratio (w/w) to poly(A)$^+$ RNA] recorded in oocytes injected with poly(A)$^+$ RNA (17 ng) from swine, human, or rat pituitary or rat hypothalamus [upward arrows [(B) through (D)] indicate ligand (1 μM MK-0677) addition; background (no signal) is ~30 counts per second (cps)]. (**D**) Aequorin light responses for the isolated GHS-R clone (7-3; 1 μM MK-0677). (**E**) Electrophysiological responses with clone 7-3 (1 ng cRNA) in response to 100 nM MK-0677. The horizontal line indicates the duration of ligand exposure.

sponsiveness of humans to GHSs (*16*). This assay was used to screen cRNA pools from a swine pituitary cDNA library for a GHS-R cDNA clone (*17, 18*). A single pool of 10,000 cRNAs (termed S10-20) was identified (out of ~2×10^6 individual cDNAs), which reproducibly gave MK-0677–evoked light responses. Stepwise fractionation resulted in the identification of a single clonal isolate (Fig. 1, D and E; clone 7-3). Supplementation with $G\alpha_{11}$ was not required for receptor bioactivity when cRNA pool complexity dropped below 50 clones per pool, possibly because a larger amount of GHS-R was expressed from these samples, which allowed even inefficient G protein coupling events to result in Ca^{2+} release.

The nucleotide sequence of clone 7-3 [~1.5 kb, including a short poly(A)$^+$ tail] revealed an open reading frame encoding a 353–amino acid protein of ~40 kD. Structural features typical of GPC-Rs include seven predicted transmembrane (TM) domains, three intracellular and three extracellular loops, and the GPC-R triplet signature sequence (Fig. 2). However, clone 7-3 appeared to be truncated at its NH_2-terminus because there was no methionine translation initiation codon located upstream of TM domain 1 [TM-1; mutagenesis showed that an aberrant translation initiation codon is most likely used (*15*)]. Additional cDNA clones were obtained from both a swine and a human pituitary cDNA library by hybridization with a radiolabeled cDNA probe derived from clone 7-3 (*19*). Nucleotide sequence analysis revealed two types of cDNAs (Fig. 2; GenBank accession numbers U60178 to U60181). The full-length human and swine type Ia GHS-R cDNAs encoded a predicted polypeptide of 366 amino acids with seven TM domains. Swine clone 7-3 belongs to this class but lacks the first 13 amino acids of the protein. The human and swine type Ib GHS-R cDNAs encoded polypeptides of 289 amino acids with only five predicted transmembrane domains. The nucleotide sequences for the human or swine type Ia and type Ib GHS-Rs are identical from the methionine translation initiation codon to Leu^{265}. Beyond Leu^{265}, the type Ib GHS-R cDNA diverges in its nucleotide sequence from the type Ia cDNA and is fused to a short conserved reading frame of only 24 amino acids, followed by a translation stop codon. In both humans and swine, the nucleotide sequence surrounding the proposed translation initiation codon (identical for type Ia and type Ib cDNAs) is in close agreement with the translation initiation consensus sequence and is preceded by an in-frame stop codon, thus assuring that the correct methionine initiation codon is identified. Both cDNA types are probably derived from a single gene by alternative mRNA processing. Genomic analysis of the gene encoding GHS-R is consistent with the presence of a single, highly conserved gene in human, chimpanzee, swine, bovine, rat, and mouse genomic DNA (*15*).

Human and swine type Ia sequences are ~93% identical and ~98% similar at the amino acid level. Type Ia and type Ib GHS-Rs share only limited amino acid sequence identity to other proteins, and



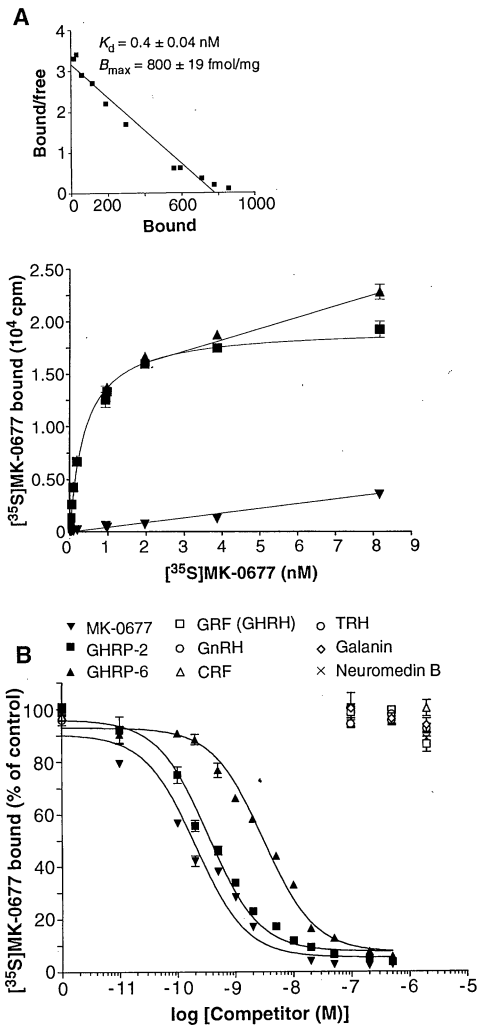


Fig. 2 (left). GHS-R predicted amino acid sequence (*25*). Type Ia and type Ib predicted amino acid sequences from swine and human GHS-R are shown. Identical residues are boxed. Conserved cysteine residues and the GPC-R signature sequence Glu-Arg-Tyr^{142} are shaded gray. Potential sites for N-linked glycosylation (arrows) and phosphorylation (asterisks) are shown. TM represents the transmembrane domains. **Fig. 3 (right).** Binding of [^{35}S]MK-0677 to crude membranes from COS-7 cells transfected with human type Ia GHS-R cDNA. (**A**) Saturation isotherm (▼, nonspecific binding; ▲, total binding; and ■, specific binding) and Scatchard analysis (inset) of [^{35}S]MK-0677 binding (bound units are femtomole per milligram of protein). (**B**) Competition analysis (0.24 nM [^{35}S]MK-0677). Competition binding data was analyzed by a nonlinear regression curve-fitting program (Prism V, version 2.0; Graph Pad Software, San Diego, California). Results shown are the means (± SEM) of triplicate determinations; K_i's were calculated by use of the formula $K_i = K_d/1 + c/K_d$.

the closest identities were to the neurotensin and TRH receptors, with ~35% and 29% identity and 59% and 56% similarity, respectively (values are for the human GHS-R open reading frame compared to human neurotensin receptor and TRH receptor; identity is centered in the transmembrane regions of the GPC-Rs). A dendrogram of the GHS-R and other GPC-Rs indicates its relatively isolated position in the neurotensin receptor and TRH receptor branches (*15*).

Full-length swine and human type Ia cRNAs expressed in oocytes responded to MK-0677 concentrations as low as 0.1 nM with a EO_{50} of 20 nM (aequorin assay). A characteristic Ca^{2+}-activated chloride current was observed in oocytes injected with the human or swine type Ia GHS-R cRNA (Fig. 1E). Peptidyl and nonpeptidyl bioactive GHSs were active in a rank order of efficacy similar to that observed for the native pituitary receptor (*12*). The NH_2-terminal truncated forms of the type Ia receptor (swine 7-3 and human 1146) were about one-tenth as active as their full-length counterparts in the aequorin oocyte assay (*15*). Type Ib cRNAs failed to give a response when injected into oocytes, even though a type Ib protein of the correct size was generated from the cRNA in vitro (*15*).

The binding of [^{35}S]MK-0677 to crude cell membranes prepared from transiently transfected COS-7 cells confirmed that type Ia cDNAs from human and swine, but not type Ib cDNAs, conferred binding that was high-affinity, saturable, and specific [binding affinity (K_d) = 0.4 nM (human type Ia GHS-R); maximum binding capacity (B_{max} = 800 fmol/mg of crude membrane protein; Fig. 3A] (*20*). Competition of radioligand by various GHSs and other ligands on the human type Ia GHS-R resulted in inhibition constant (K_i) values of 0.1 nM for MK-0677, 1.9 nM for GHRP-6, and 0.21 nM for GHRP-2 with a rank order of potency that is consistent with each of their GHS activities. Galanin, GHRH, GnRH, corticotropin-releasing factor (CRF), neuromedin B, and TRH failed to compete with the radioligand (Fig. 3B). Similar results were obtained for the swine GHS-R. We conclude that the pharmacological profile of GHS binding to cloned human and swine type Ia GHS-Rs correlates with their biological activities in vivo.

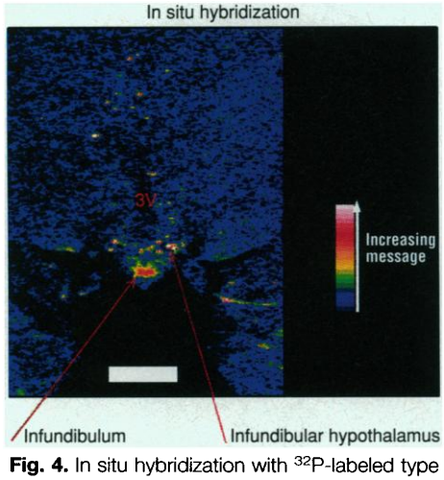


Fig. 4. In situ hybridization with ^{32}P-labeled type Ia–specific GHS-R oligonucleotide probes in rhesus brain (29-day exposure; serial coronal sections of whole rhesus brain from three monkeys were analyzed). An example of the hypothalamic region in which positive signals were observed is shown. The third ventricle is indicated by 3V; the white bar equals 0.5 cm.

Functional assessment of sucrose gradient–fractionated poly(A)$^+$ RNA from swine pituitary gland in oocytes gave a single size class of GHS-R activity (1.6 to 2.3 kb). Abundance of GHS-R cDNA measured about 1 in roughly 300,000, as calculated from polymerase chain reaction (PCR) amplification of GHS-R sequences in pools of an unamplified swine pituitary gland library. Ribonuclease (RNase) protection or functional assays specifically confirmed the presence of human GHS-R mRNA in pituitary gland, whereas the mRNA could not be detected in whole brain, liver, placenta, spleen, and kidney. GHS-R mRNA could also be detected in the hippocampus by RNase protection (*15*). Type Ia GHS-R mRNA was identified in the arcuate-ventromedial hypothalamus and infundibular hypothalamus by in situ hybridization (Fig. 4) (*21*, *22*). Expression of GHS-R on neurons near the median eminence supports the proposed role of the GHS-R as a critical component of a neuroendocrine pathway involved in pulsatile GH release.

The molecular characterization of this GPC-R excludes the idea that the GHSs act on an alternatively spliced form of a previously characterized GPC-R involved in the neuroendocrine control of GH release. The characterization of this GPC-R provides evidence for the presence of an endocrine pathway distinct from that described for GHRH and somatostatin that contributes to the control of GH release. The GHSs appear to act on the pituitary gland and the arcuate ventromedial and infundibular hypothalamus (Fig. 4) (*23*, *24*), and the presence of GHS-R may represent a regulatory feedback loop involved in the control of GHRH and somatostatin release. Type Ia GHS-R may thus contribute to the control of pulsatile GH release, presumably under the influence of an unidentified endogenous ligand.

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17. Total RNA was isolated from swine (50 to 80 kg body weight, Yorkshire strain) pituitary glands and other tissues (frozen in liquid nitrogen 1 to 2 min after animals were killed) as described [P. Chomczynski and K. Mackey, *Anal. Biochem.* **225**, 163 (1993)]. We synthesized cDNA from poly(A)$^+$ RNA as per the manufacturer's instructions (Superscript, Gibco-BRL, Gaithersburg, MD) with an oligo(dT)–Not I primer-adapter. Double-stranded cDNA was ligated to Eco RI adapters and digested with Not I. Large cDNA was isolated on a Sephacryl S-500 column (Pharmacia) and ligated to pSV-7. We constructed pSV-7 by expanding the multiple cloning site in pSG-5 [Stratagene, La Jolla, CA; S. Green, I. Isseman, E. Sheer, *Nucleic Acids Res.* **16**, 369 (1988)]. The library was solid-state amplified after separation into pools of 500 to 1000 cDNAs per pool [*E. coli* DH10B; ~3 × 10^6 independent clones; 95% inserts; insert size ~1.65 kb; M. P. Kriegler, *Gene Transfer and Expression: A Laboratory Manual* (Stockton Press, New York, 1990)]. We synthesized cRNA from Not I–digested DNA by using, with modifications, a kit from Promega Biotech [J. P. Arena *et al.*, *Mol. Pharmacol.* **40**, 368 (1991)].
18. For the aequorin assay, library pool cRNA (500 ng/ml in RNase-free water; 25 ng per oocyte for pool sizes of 500 to 10,000), aequorin cRNA (2 ng per oocyte), and $G\alpha_{11}$ cRNA (2 ng per oocyte) were injected. Expression of GHS-R was detected as early as 18 hours after injection and was attenuated after 72 hours. Aequorin and $G\alpha_{11}$ were transcribed from a modified expression vector [pcDNA-3v2, derived from pcDNA-3 (InVitrogen)] by insertion of a Bgl II, dA(20), Sfi I cassette (5′ to 3′) to append a poly(A) tract on cRNAs [$G\alpha_{11}$ released as a Cla I to Not I fragment from the pCMV vector; D. Wu *et al.*, *J. Biol. Chem.* **266**, 9309 (1992); the ORF of the aequorin cDNA [S. Inouye *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **82**, 3154 (1985)] was removed from pCDM.aeq by PCR [D. Button and M. Brownstein, *Cell Calcium* **14**, 663 (1993)] and ligated into pcDNA-3v2 with an optimized Kasak translational initiation sequence. *Xenopus* oocytes (Xenopus One, Ann Arbor, MI) were prepared and injected, and the membrane currents were recorded as described [J. P. Arena *et al.*, *Mol. Brain Res.* **15**, 339 (1992)]. Electrophysiological recordings were made 2 to 5 days after injection. We measured bioluminescent responses with a Berthold Luminometer LB953 connected to a PC running the Autolumat-PC Control software (Wallac, Gaithersburg, MD). Batches of oocytes from three to five frogs were tested and selected on the basis of their ability to express a control G protein–linked receptor (human GnRH receptor) and their ability to show robust 1% chick serum–induced phospholipase C activation [R. Grygorczyk *et al.*, *J. Neurosci. Methods*, in press]. Oocytes from one to two frogs were chosen for cRNA injection [50 nl of cRNA for the equivalent of 25

ng (complex pools) to 0.5 ng (pure clone) per oocyte]. Oocytes were incubated for 24 to 48 hours, treated with coelenterazine [10 μM coelenterazine (Molecular Probes, Eugene, OR) and 30 μM reduced glutathione in OR-2 media (no calcium)] for 2 to 3 hours with gentle orbital shaking in the dark at 18°C, and returned to ND-86 medium with calcium (maintained in the dark with shaking) until measurements were initiated. For luminometer measurements, oocytes (singly or in pairs) were transferred to plastic tubes (75 mm by 12 mm, Sarstedt) containing 2.9 ml of Ca^{2+}-free OR-2 medium. Each cRNA pool was tested in triplicate. Measurements (duration 2 min) were triggered by the injection of 0.1 ml of 30 μM MK-0677.

19. Additional GHS-R clones from the swine cDNA library were identified by hybridization of the clone 7-3 ^{32}P-labeled insert to slot-blot pools of plasmid DNA (500 cDNAs per pool). Filters were prehybridized [at 42°C for 4 hours in 5× standard saline citrate (SSC) with 5× Denhardt's solution, 250 μg/ml of tRNA, 1% glycine, 0.075% SDS, 50 mM $NaPO_4$ (pH 6), and 50% formamide], and hybridizations were done at 42°C for 20 hours in 5× SSC, with 1× Denhardt's solution, 0.1% SDS, 50 mM $NaPO_4$, and 50% formamide. Clonal isolates were identified by colony hybridization. Human pituitary homologs of the swine GHS-R were obtained by screening a cDNA library [lambda ZAP II (Stratagene); ~2×10^6 phages gave 21 GHS-R clones]. DNA sequencing was done on both strands [automated Applied Biosystems instrument (ABI model 373); manually by dideoxy chain termination (Sequenase version 2.0; U.S. Biochemical, Cleveland, OH)]. Database searches [GenBank 92, EMBL 43, Swiss-Prot 31, PIR 45, dEST (Gbest 92), and Prosite 12], sequence alignments, and analysis of the GHS-R nucleotide and protein sequences were done with the GCG Sequence Analysis Software (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).
20. We transfected COS-7 cells with GHS-R expression plasmids by using lipofectamine (Gibco-BRL) as described. Binding of [^{35}S]MK-0677 (~1000 Ci/mmol) was done with control swine pituitary membranes, membranes from mock transfected cells, and crude membranes prepared from transfected cells (*12*). Crude cell membranes were prepared on ice at 48 hours after transfection. Binding reactions were done at 20°C for 1 hour in a total volume of 0.5 ml containing 0.1 ml of membrane suspension (25 μg of protein), 10 μl of [^{35}S]MK-0677 (0.05 to 1 nM), 10 μl of competing drug, and 380 to 390 μl of homogenization buffer. Specific binding (>90% of total) equaled the difference between total and nonspecific binding obtained in the presence of 50 nM unlabeled MK-0677.
21. Rhesus monkeys were euthanized, and the brains were removed and immediately frozen in isopentane on dry ice at −35°C and stored at −70°C. Coronal or sagittal sections (~10 μm) were cut in a cryostat (Reichert) at −18°C to −20°C. Sections were thaw-mounted on "Probe On" slides (Fisher Scientific), air-dried for approximately 1 hour, fixed in 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS) (pH 7.4) for 5 min, rinsed in PBS for 2 min, defatted and dehydrated in an ethanol series (50, 70, and 95% for 5 min each), and stored in 95% ethanol at +4°C. The 3′ end–labeled probes, specific for the type Ia human GHS-R (specific activity ~1.5×10^9 cpm/μg), were each 45 bases long and antisense to nucleotides 855 through 909 and 979 through 1023. Hybridizations of rhesus brain sections were done as described [D. J. S. Sirinathsinghji *et al.*, *Neuroscience* **34**, 675 (1990); D. J. S. Sirinathsinghji and S. B. Dunnet, in *Molecular Imaging in Neuroscience*, N. Sharif, Ed. (Oxford Univ. Press, Oxford, 1993), p. 43]. After hybridization, the sections were washed for 1 hour in 1× SSC at 57°C, briefly rinsed in 0.1× SSC and dehydrated in 70% and 95% ethanol, air-dried, and then exposed to Hyperfilm β-max x-ray film (Amersham) for 7 days. Adjacent slide-mounted sections incubated with labeled oligonucleotide probe in the presence of a 100-fold excess of unlabeled oligonucleotide probe or with a sense probe from the same region produced no hybridization signal.
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25. Abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
26. We thank B. DeFranco, K. Judd, E. Szekely, and D. Thompson (Branchburg Farm) for swine tissues; D. Boltz and A. Sirotina for discussion and fluorescence-activated cell sorting analysis; D. Button and M. Brownstein (NIH) for aequorin cDNAs; C. Y. Bowers (Tulane Medical School) for GHRP-2; K. Elliston for sequence analysis; D. Leong for helpful discussion; K. Prendergast and D. Underwood for helpful discussion and modeling of the GHS binding site; Y. Karkhanis for antibody analysis; J. Ngai, T. Livelli, and colleagues (Molecular Cell Sciences) for support in the production of cDNA libraries and advice; K. Likowski for expert secretarial assistance; and MRL Visual Communications for preparation of figures. The care of animals was in accordance with institutional guidelines.

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TECHNICAL COMMENTS

High-Affinity Potassium Uptake in Plants

Plant roots accumulate K^+ against its electrochemical gradient from the micromolar amounts that prevail in most soils (*1*). Francisco Rubio *et al.* (*2*) elegantly demonstrate that the high-affinity K^+ uptake transporter HKT1 from wheat roots functions as a Na^+-coupled cotransporter when expressed in yeast or *Xenopus* oocytes. Energization could therefore be provided by an inwardly directed electrochemical gradient for Na^+ across the plasma membrane, as is the case in some aquatic species (*3*).

We evaluated the possibility that Na^+-coupled K^+ transport comprises the major route for K^+ absorption in intact K^+-starved wheat roots using an electrophysiological and a radiometric approach. Addition of micromolar K^+ (or Rb^+ or Cs^+) induced marked membrane depolarizations (Fig. 1), which are typical of high-affinity transport (*4*) and absent in non-K^+–starved plants. However, depolarizations occurred in the effective absence of Na^+ and were indifferent to the presence of 1 mM Na^+. Then we tested high-affinity unidirectional uptake of the K^+ analog Rb^+ for its Na^+ dependence. Na^+ did not stimulate uptake and may even have inhibited it (Table 1).

To examine whether induction of Na^+-coupled K^+ transport requires previous exposure to Na^+ or whether it could be derepressed by relative nonavailability of H^+ for K^+:H^+ symport, we grew plants in the presence of 1 mM Na^+ or at pH 9. In each case only Na^+-independent, high-affinity K^+ and Rb^+ uptake was observed.

In situ hybridization showed that *HKT1*

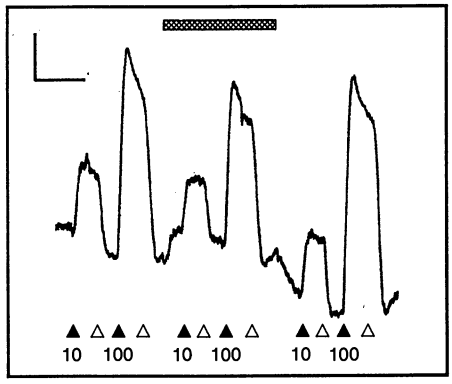

Fig. 1. Representative example of a trace showing the effect of external K^+ on the electric potential at the root surface of wheat. Wheat cultivar Atlas 66, the original source of *HKT1*, was starved of K^+ for 6 days and grown on 1 mM $CaCl_2$ at pH 6, in the presence of 1.0 mM Na^+. Closed symbols denote addition of 10 or 100 μM K^+, respectively. Open symbols denote washout of K^+. Hatched bar at the top indicates the presence of 1 mM Na^+. Horizontal and vertical scale bars are 50 s and 10 mV, respectively. Solutions were freshly prepared, and all experimental treatment was carried out in plastic to avoid Na^+ contamination. Measurements shown were made 0 to 5 mm from the root tip.

Table 1. Unidirectional Rb^+ influx (μmol·gFW^{-1}·hour^{-1}) into roots of wheat (cv. Maris Dove). Plants were grown at pH 6 with or without 1 mM Na^+, and the external Rb^+ was 50 μM. Values are the mean ± SEM of three independent determinations. Solutions were freshly prepared, and all experimental treatment was carried out in plastic to avoid Na^+ contamination.

Solution	Na^+ concentration in uptake buffer (μM)				
	0	20	50	150	500
(Minus Na^+)	2.04 ± 0.06	2.00 ± 0.05	2.14 ± 0.06	2.00 ± 0.14	1.61 ± 0.20
(Plus Na^+)	1.64 ± 0.07	1.48 ± 0.03	1.50 ± 0.10	1.62 ± 0.20	1.68 ± 0.12