parable with those of the replacements at site 44. The χ^1 values of Val^{44}, Ile^{44}, and Thr^{44} are, respectively, 164°, −73°, and −66°; all are close to an expected energy minimum. It was argued on the basis of Ala, Val (χ^1 = 162°), and Thr (χ^1 = −76°) substitutions at site 131 that β branched amino acids might be helix-destabilizing because of the strain introduced, although the apparent strain energy was not large (*17*). The possible involvement of strain in such cases remains an open question.

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22. It is expected that the helix propensity of Gly is less than that predicted from hydrophobic stabilization alone because of the increase in backbone configurational entropy associated with this amino acid (*27, 28*). (This decrease in helix propensity for Gly is indicated by the dotted vertical line in Fig. 2.) That the helix propensity of Ala is greater than that expected on the basis of hydrophobic stabilization alone (shown by the vertical dotted line in Fig. 2) can also be rationalized in terms of the lack of a γ carbon in Ala and the consequently reduced side chain configurational entropy (*8, 17, 27*). Thus, the remaining outliers are Arg, Phe, and Trp, which all have long, bulky side chains. In the crystal structure, the Arg^{44} side chain adopts an unusual (nonextended) conformation (Fig. 1A) such that two of the guanidino nitrogens form hydrogen bonds with the side chain of Asn^{116} of a neighboring molecule in the crystal lattice. (The ε amino group of Lys^{44} participates in a similar intermolecular interaction in the Lys^{44} crystal structure.) If it is assumed that the side chains of Arg^{44} and Lys^{44} adopt fully extended conformations in solution, then the estimated hydrophobic stabilization of each of these residues corresponds moderately well with its observed helix propensity (● → ○, in Fig. 2). The buried surface areas of the bulky residues Phe and Trp do not appear to correspond to their helix propensities (Fig. 2). However, in both cases there were complications in the structural analysis that might affect the calculation of surface area. The Phe^{44} variant crystallized in a form different from that of the wild type with two molecules per asymmetric unit. The Trp^{44} variant crystallized in yet another form with four molecules per asymmetric unit. All six of these mutant lysozyme molecules have Phe^{44} or Trp^{44} in the trans conformation ($\chi_1 \sim 180°$) (Fig. 1B). This conformation is the same as that of Ser^{44} in the native protein but is different from all the other position-44 variants, which are gauche$^+$ ($\chi_1 \sim -60°$). Because they adopt the trans conformation, Phe^{44} and Trp^{44} are close to Glu^{45} and to Lys^{48} in the next turn of the α helix. This proximity helps to explain why the calculated buried surface area of Phe^{44} and Trp^{44} is so much larger than that of the other position-44 replacements (Fig. 2). On the other hand, the contacts between either Trp^{44} or Phe^{44} and residues 45 and 48 are tenuous. In the crystal structures of these variants, there are on average only 2.6 van der Waals contacts of less than 3.5 Å between any side chain atom in residue 44 and any side chain atom in residues 45 and 48. On the assumption that in solution Glu^{45} and Lys^{48} do not reduce the solvent-accessible areas of Phe^{44} and Trp^{44}, the hydrocarbon surface area that these two residues would then bury is close to their observed hydrophobic stabilization (● → ○ in Fig. 2).
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30. The buried area was determined as the difference between the folded and the unfolded solvent-accessible surfaces (*29*) of the residue 44 side chain. The unfolded state for each mutant was modeled by a fully extended tripeptide (φ = −139°, ψ = 135°) (*19*) with the same sequence (Lys-X-Glu) as residues 43 to 45. The side chain at site 44 was taken to be fully extended and the calculated solvent accessibility was taken as the average of the values for the gauche$^+$, gauche$^-$, and trans conformations. For each mutant, the folded protein was taken as the protein that was observed in the respective crystal structure. Only the hydrocarbon surface area (all carbon atoms plus S^γ of Met) was included in the calculation. The radius of the probe used for the calculations was 1.4 Å. The straight line was fitted by linear regression to the points for Asn, Glu, Ser, Thr, Val, Lys, Ile, and Leu (correlation coefficient = 0.96).
31. The solvent-accessible surface area that is buried upon helix formation should be small (less than that of a methyl group) and the corresponding hydrophobic stabilization is also small (less than 1 kcal mol^{-1}). Estimates of buried surface area are therefore sensitive to changes in side chain conformation, geometry of the α helix, and the model used in the estimation of the solvent-exposed area in the unfolded state. In an attempt to minimize these sources of uncertainty, we based the surface area calculation only on the side chains of the substituted residue. Calculations of surface area based on the helix as a whole, as well as attempts to estimate entropic effects, will be described elsewhere (M. Blaber *et al.*, in preparation).
32. Abbreviations: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; Y, Tyr.
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A Nonpeptidyl Growth Hormone Secretagogue

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A nonpeptidyl secretagogue for growth hormone of the structure 3-amino-3-methyl-*N*-(2,3,4,5-tetrahydro-2-oxo-1-{[2′-(1*H*-tetrazol-5-yl)(1,1′-biphenyl)-4-yl]methyl}-1*H*-1-benzazepin-3(*R*)-yl)-butanamide (L-692,429) has been identified. L-692,429 synergizes with the natural growth hormone secretagogue growth hormone–releasing hormone and acts through an alternative signal transduction pathway. The mechanism of action of L-692,429 and studies with peptidyl and nonpeptidyl antagonists suggest that this molecule is a mimic of the growth hormone–releasing hexapeptide His-D-Trp-Ala-Trp-D-Phe-Lys-NH_2 (GHRP-6). L-692,429 is an example of a nonpeptidyl specific secretagogue for growth hormone.

The availability of recombinant human growth hormone (GH) has resulted in renewed interest in GH therapy. Aging is associated with an attenuation of the amplitude and frequency of pulsatile GH release; by age 40, a reduced responsiveness of the pituitary gland to GH-releasing hormone (GRF) is evident (*1*). GH replacement therapy may reverse some of the bodily changes associated with aging (*2*); thus, GH treatment may prove beneficial to the elderly. In addition to its utility in the treatment of GH-deficient children, GH accelerates healing of severely burned children (*3*), is beneficial in preventing catabolic side effects in patients treated with prednisone (*4*), acts as an adjuvant to gonadotrophin treatment for ovulation induction (*5*), may prevent osteoporosis (*6*), and improves exercise capacity in GH-deficient adults (*7*). GH also stimulates T cell development, which suggests that it may be useful for the treatment of T cell deficiencies (*8*).

Current methods of treating GH deficiency are not ideal. GH, a large polypeptide, lacks oral bioavailability, and injection results in prolonged elevation of GH

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concentration. GH is normally released in a circadian, pulsatile fashion. This release is controlled by two hypothalamic hormones: GRF, which induces GH release, and somatostatin, which is inhibitory. Whereas appropriately administered GRF could produce a pulsatile release of GH and thereby possibly avoid the side effects caused by persistent GH elevation, as a peptide GRF lacks good oral bioavailability. For an alternative to GH and GRF as drugs, we sought a small nonpeptide molecule suitable for oral administration that would function as a specific GH secretagogue. Nonpeptide mimetics provide the structural diversity necessary to allow a molecule to be optimized for specificity, oral bioavailability, and pharmacokinetic properties. An orally active secretagogue would be useful for GH replacement because it would permit GH release to mimic that in normal physiology.

Consideration of the structure-activity relations of the peptidyl GH secretagogue GHRP-6 (His-D-Trp-Ala-Trp-D-Phe-Lys-NH_2), described by Bowers and Momany and co-workers (9, *10*), led us to conclude that the aromatic amino acid residues and the NH_2-terminal amine were important for bioactivity. On the basis of this hypothesis, we selected a diverse group of structures that contained these features and tested their capacity to specifically induce GH release in rat pituitary cultures. We initially identified L-158,077 (Fig. 1), which had modest potency in the pituitary cell assay [median effective concentration (EC_{50}) = 3 μM] but was specific for inducing GH secretion (*11*). By substituting the carboxylic acid function with a tetrazole (*12*), we achieved a dramatic increase in potency (L-158,432; Fig. 1) (EC_{50} = 120 nM). Resolution of the *R* and *S* enantiomers established the *R* configuration (L-692,429; Fig. 1) as the active enantiomer (EC_{50} = 60 nM; Fig. 2A) (L-692,428, *S* enantiomer; EC_{50} > 10 μM).

L-692,429 has many properties similar to those of GHRP-6. Antagonism of L-692,429 by the structurally related non-GH secretagogue L-692,400 (Fig. 1) is shown in Fig. 2B. L-692,400 also antagonized GH stimulation caused by GHRP-6 but did not antagonize GRF (Fig. 2, C and D). Similarly, the peptidyl antagonist related to GHRP-6, His-D-Trp-D-Lys-Trp-D-Phe-Lys-NH_2, was also a preferential antagonist of GHRP-6 and L-692,429. Furthermore, in combination with concentrations of GHRP-6 that maximally stimulated GH release, L-692,429 produced no further increase in GH secretion. However, in combination with GRF, L-692,429 caused a synergistic increase in GH secretion (Fig. 2E) that was accompanied by a similar effect on amounts of intracellular adenosine 3′,5′-monophosphate (cAMP). GHRP-6

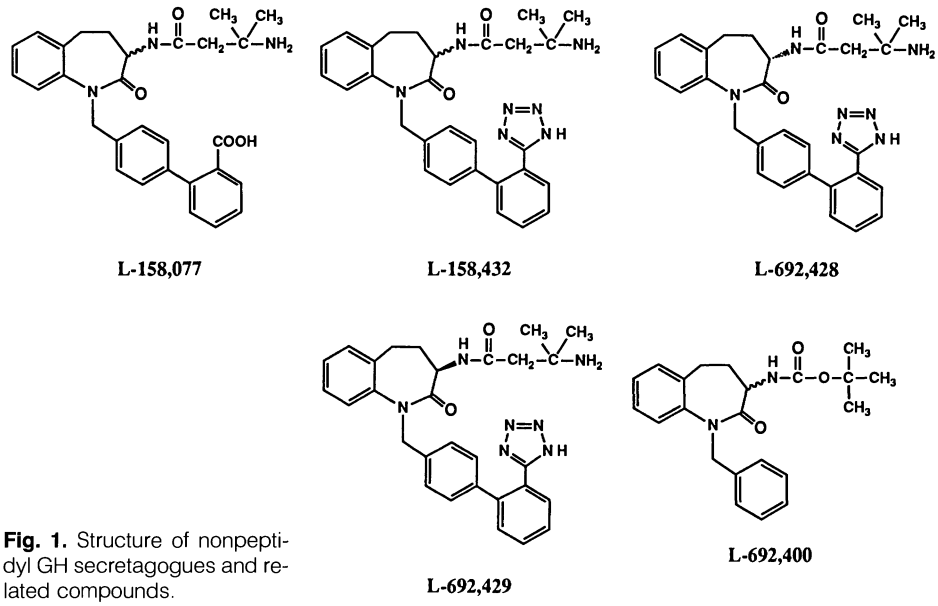

Fig. 1. Structure of nonpeptidyl GH secretagogues and related compounds.

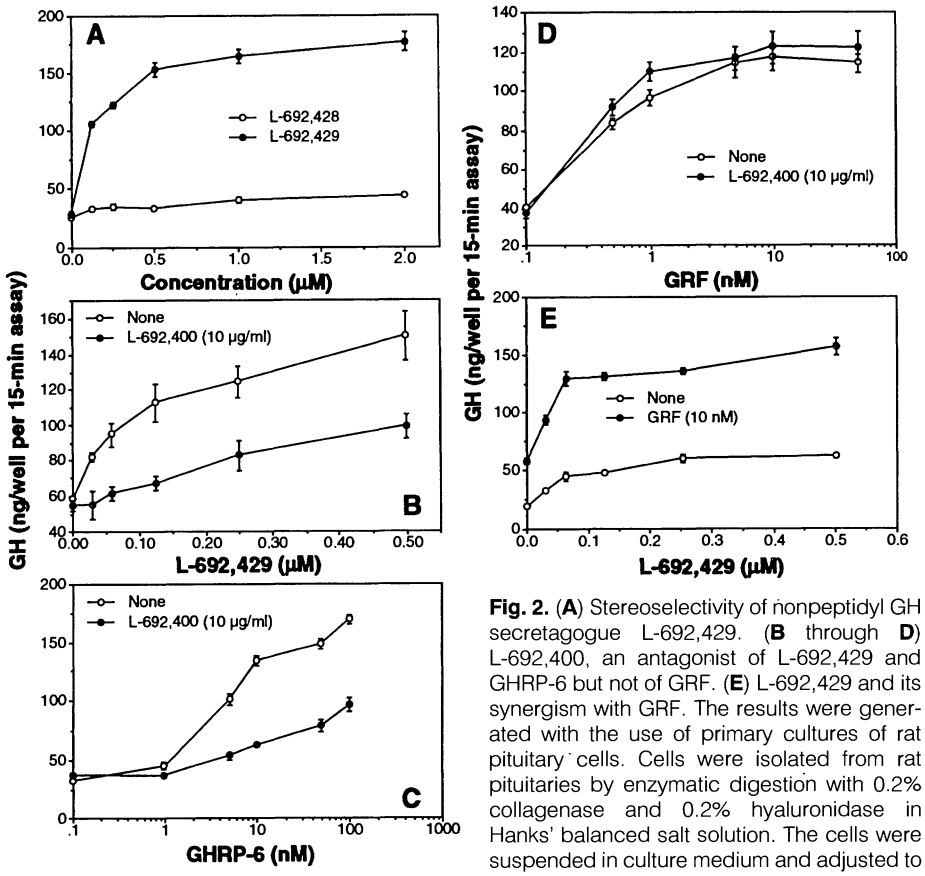

Fig. 2. (**A**) Stereoselectivity of nonpeptidyl GH secretagogue L-692,429. (**B** through **D**) L-692,400, an antagonist of L-692,429 and GHRP-6 but not of GRF. (**E**) L-692,429 and its synergism with GRF. The results were generated with the use of primary cultures of rat pituitary cells. Cells were isolated from rat pituitaries by enzymatic digestion with 0.2% collagenase and 0.2% hyaluronidase in Hanks' balanced salt solution. The cells were suspended in culture medium and adjusted to a concentration of 1.5×10^5 cells per milliliter, and 1.0 ml of this suspension was placed in each well of a 24-well tray. Cells were maintained in a humidified 5% CO_2–95% air atmosphere at 37°C for 3 to 4 days. The culture medium consisted of Dulbecco's modified Eagle's medium containing 0.37% $NaHCO_3$, 10% horse serum, 2.5% fetal bovine serum, 1% nonessential amino acids, 1% glutamine, 1% nystatin, and 0.1% gentamycin (*13*). Before testing compounds for their capacity to cause GH release, we washed cells twice 1.5 hours before and once more immediately before the start of the experiment with the above culture medium containing 25 mM Hepes (pH 7.4). We tested compounds in quadruplicate by adding them in 1 ml of fresh medium to each well and incubating them at 37°C for 15 min. After incubation, the medium was removed and centrifuged at 2000*g* for 15 min to remove any cellular material. The supernatant fluid was removed and assayed for GH by radioimmunoassay.

behaved identically (*13*). For treatment of GH deficiency, synergy with GRF would be an obvious advantage.

Additional findings indicated that L-692,429 and GHRP-6 act through a similar site. We have shown with static (*13*) and perifusion studies (*14*) that after GHRP-6 stimulates GH release, rat pituitary cells become temporarily desensitized to subsequent stimulation by GHRP-6. The kinetics of L-692,429–stimulated GH release and desensitization paralleled those of GHRP-6 (*15*). Moreover, cells desensitized to either L-692,429 or GHRP-6 were insensitive to subsequent stimulation by both secretagogues, although the cells remained responsive to GRF.

With the fluorescent dye bis-oxonol, we have shown that L-692,429, like GHRP-6, causes depolarization of pituitary cells (*16*). With the use of a variety of pharmacologic agents to depolarize the cells, we also have shown that depolarization causes increases in GH release and results in a synergy with GRF identical to that observed with GHRP-6 (*16*) and L-692,429. To directly define the action of L-692,429 on somatotrophs, we used a reverse hemolytic plaque assay (*17*) to identify somatotrophs and subjected these cells to fluorescence ratio imaging and electrophysiological studies. Fluorescence ratio imaging showed that L-692,429, but not its inactive enantiomer L-692,428, caused an increase in the concentration of intracellular Ca^{2+} (Fig. 3). This increase was blocked by nifedipine and Ω Agatoxin IIIA but not by ω-conotoxin, which is consistent with an effect on L-type Ca^{2+} channels. These specific Ca^{2+} channel blockers also inhibited L-692,429–mediated GH release. Electrophysiology showed that GHRP-6 and L-692,429 blocked K^+ currents, which resulted in depolarization of somatotrophs (*18*).

A common second messenger pathway is shared by both GHRP-6 and L-692,429. Both secretagogues appeared to mediate GH release by way of protein kinase C (*19*). The synergistic effects of GHRP-6 and L-692,429 on GH release and cAMP production were mimicked by phorbol 12-myristate 13-acetate (PMA) treatment. Exposure of pituitary cells to PMA for 24 hours dramatically attenuated the effects of GHRP-6 without affecting their responsiveness to GRF (*15*); L-692,429 behaved identically.

A dose-dependent increase in serum GH similar to that observed with GHRP-6 (*10, 20*) occurred when L-692,429 was administered to rats, dogs, sheep, pigs, rhesus monkeys, and humans (*21*). Figure 4 illustrates the specificity of GH release in three male beagles. Plasma amounts of GH, luteinizing hormone (LH), triiodothyronine (T3), thyroxine (T4), prolactin, insulin, adrenocorticotropic hormone (ACTH), and cortisol were measured. Except for slight increases in ACTH and cortisol, the other hormones were unaffected by treatment with L-692,429, which demonstrates the specificity of L-692,429 as a GH secretagogue.

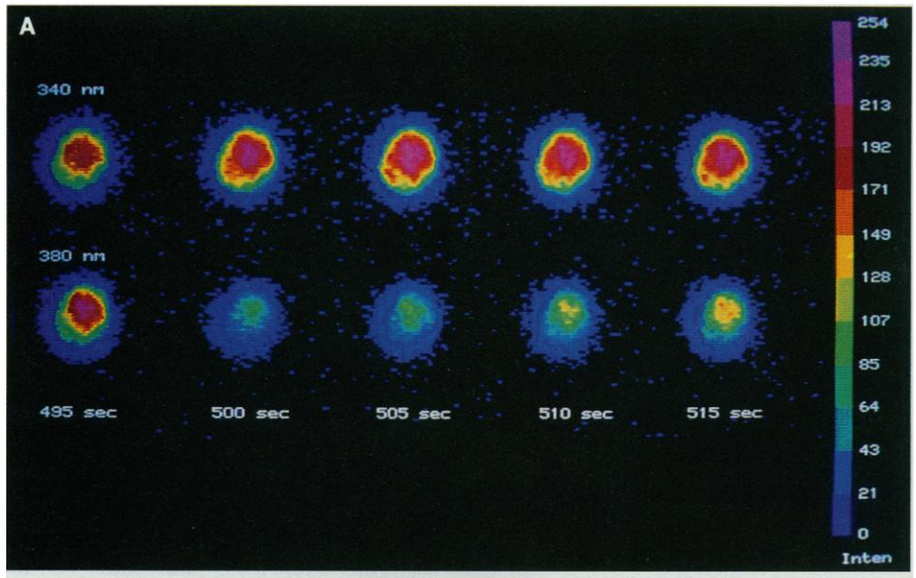

Fig. 3. Effect of L-692,429 and its enantiomer L-692,428 on cytosolic free Ca^{2+} concentration of rat somatotrophs as determined by fluorescence ratio imaging. Images of a GH-secreting cell at 340 nm and 380 nm were taken after addition of L-692,428 and L-692,429. (**A**) Images after addition of L-692,429 as a function of time; no changes were observed after the addition of the inactive enantiomer L-692,428. (**B**) Graphic representation of the concentration of free Ca^{2+} in response to L-692,428 and L-692,429 (*24*).

The activity and specificity of L-692,429 across species suggests that there is a physiologically relevant natural ligand that has an undefined function in the regulation of GH secretion. A number of different molecules are known to affect GH levels in vivo or in vitro, although none of them precisely mimics L-692,429. The effects of somatostatin, adenosine, cholecystokinin, insulin, potassium channel antagonists, 5-hydroxytryptamine agonists, substance P, sodium channel agonists, calcium channel agonists, thyrotropin-releasing hormone, vasoactive intestinal peptide, α_2-adrenergic agonists, muscarinic agonists, dopamine, γ-aminobutyric acid, glutamate, galanin, and Met-enkephalin were compared with L-692,429. At concentrations up to 10 μM, L-692,429 showed no significant binding either to the receptors of these ligands or to specific ion channels. Electrophysiological experiments showed that like GHRP-6 (*18*), L-692,429 behaves as a functional somatostatin antagonist in

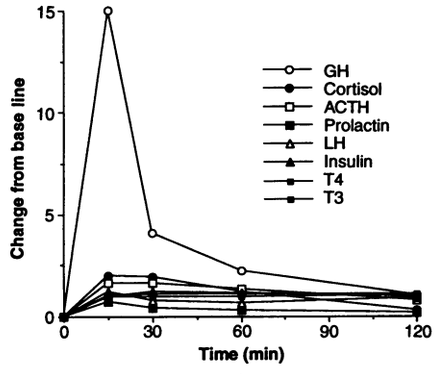

Fig. 4. Specificity of L-692,429 as a GH secretagogue. Three male beagles (13 to 16 months of age) were fasted for 15 hours before intravenous administration of L-692,429 by way of the cephalic vein. L-692,429 (0.25 mg per kilogram of body weight) was injected in physiological saline (2 ml/kg) over a 5-min period. Blood samples were collected sequentially from the jugular vein for up to 120 min and were assayed with radioimmunoassays specific for GH, cortisol, ACTH, prolactin, LH, insulin, T4, and T3. The results are expressed as means of the fold increase in plasma hormone amounts relative to pretreatment plasma amounts. The base line and peak GH concentrations after 15 min for each dog were 1.04 and 3.40, 1.76 and 25.48, and 2.00 and 53.92 ng/ml, respectively (paired *t* test; $P = 0.002$).

that it caused depolarization of somatotrophs, whereas somatostatin caused hyperpolarization. The specificity of L-692,429 has been evaluated in more than 50 receptor binding assays and more than 20 functional assays in which known ligands stimulate a biochemical or biological response. With the exception of weak angiotensin II receptor binding (concentration required for 50% inhibition was 6 μM), L-692,429 is inactive at <10 μM.

Selective nonpeptidyl kappa receptor agonists are known (*22*), although few other examples of potent and specific nonpeptidyl mimetic agonists and antagonists of peptide ligands exist (*23*). Structural modifications of L-692,429 and establishment of a structure-activity relationship as agonists or antagonists provide new and important clues to the design of nonpeptidyl mimics of peptides. By the substitution of pharmacophores, structural elements that elicit bioactivity, small molecules can be tailored to fit the receptor sites of other peptides. The advantage of such mimetics is that their structures can be readily modified either subtly or dramatically to provide the structural diversity necessary to optimize molecules suitable as oral drugs.

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24. After 3 days in cultures, rat pituitary cells were dispersed with trypsin (0.25 mg/ml) and GH-secreting cells were identified by a reverse hemolytic plaque assay (*17*). The cells attached to a glass cover slip were loaded with 1.5 μM fura-2/AM (Calbiochem) for 30 min at 37°C in 0.1% BSA-DMEM (bovine serum albumin–Dulbecco's modified Eagle's medium). The cells were then rinsed with Hanks' solution containing 10 mM Hepes (pH 7.4) and 0.1% BSA and were given 5 min to allow fura-2/AM de-esterification. The glass cover slip was mounted on a Micro-Incubator with a temperature control set at 37°C (Medical System Corp., Greenvale, NY) and placed on the Nikon microscopic stage of a Deltascan 4000-52 ratio imaging system equipped with dual excitation monochromators (Proton Technology International, South Brunswick, NJ). The fluorescence emission of a somatotroph was monitored at 510 nm with a Hamamatsu intensified charged-coupled device camera. The cytosolic free Ca^{2+} concentration was measured by the ratio of fluorescence excited by 340 nm to that excited by 380 nm and was calibrated with the equation developed by G. Grynkiewicz, M. Poenie, and R. Y. Tsein [*J. Biol. Chem.* **260**, 3440 (1985)]. Values for R_{min} (where R is the fluorescence ratio) were obtained from measurements with a solution of 20 μM fura-2 and 10 mM EGTA. R_{max} was measured after addition of 20 μM ionomycin. The R_{max}/R_{min} value was approximately 30. The results shown were obtained with a concentration of L-692,429 that caused a maximal release of GH (2 μM). Of nine somatotrophs tested, eight responded to L-692,429 with the concentration of Ca^{2+} increasing from 102 ± 34 (SE) to 516 ± 274 nM; no increase was evident with L-692,428.
25. The Ω Agatoxin IIIA is a gift from M. Smith (Merck Research Laboratories).

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Unidirectional Spread of Secondary Sexual Plumage Traits Across an Avian Hybrid Zone

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Theory predicts that traits under positive selection can rapidly cross a hybrid zone in spite of a substantial barrier to neutral gene flow between hybridizing taxa. An avian hybrid zone between *Manacus candei* (white-collared manakin) and *M. vitellinus* (golden-collared manakin) is reported here that displays an unusual pattern of noncoincident clines. Male secondary sexual traits of *M. vitellinus* have spread into populations that are genetically and morphometrically like *M. candei*. These birds have a lek breeding system in which male mating success is highly skewed, suggesting that sexual selection is driving male sexual traits across the zone.

Hybrid zones provide insight into evolutionary forces that subdivide populations and species and influence the spread of alternative adaptations (*1*). The tension zone model proposes that stability of narrow hybrid zones is maintained through a balance between selection against hybrids and dispersal of parental types across range boundaries (*2*). Selection may act against hybrid individuals because they have disadvantageous combinations of coadapted gene complexes from each parental taxon (*1*, *3*). Such selection could create a barrier to gene flow for neutral alleles linked to negatively selected loci and produce the commonly observed pattern of narrow, concordant shifts in diagnostic characters across hybrid zones. However, mathematical models predict that alleles under positive selection can spread rapidly across hybrid zones (*4*). Thus, hybrid zones may serve as evolutionary conduits between locally adapted populations, through which generally advantageous traits can spread despite a barrier to overall gene flow across the zone.

Birds in the genus *Manacus* are small, frugivorous inhabitants of lowland tropical rainforest from Mexico to Argentina. Highly sexually dimorphic, they have a lek breeding system in which the brightly colored males display at court assemblages nearly year round. The olive-colored females visit a lek to select a mate, then take sole responsibility for nesting and raising the young. Males compete for females by performing courtship displays. Female mate choice is nonrandom, few males receiving most of all copulations (*5*).

Males of *Manacus* have a black cap contrasting with a bright collar, throat, and feather beard. The species differ most obviously in the color of the throat, collar, and underparts. Male courtship display accentuates the contrast between the cap and collar by thrusting of the head and neck forward and extending of the beard.

When surveyed in Bocas del Toro Province, Panama (*6*), white-collared birds typical of *M. candei* were found only in northwestern Bocas del Toro at localities 2 and 3 (Fig. 1). Near locality 3, the Río Teribe

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