Identification of Asn289 as a Ligand Binding Site in the Rat Thyrotropin-Releasing Hormone (TRH) Receptor As Determined by Complementary Modifications in the Ligand and Receptor: A New Model for TRH Binding†

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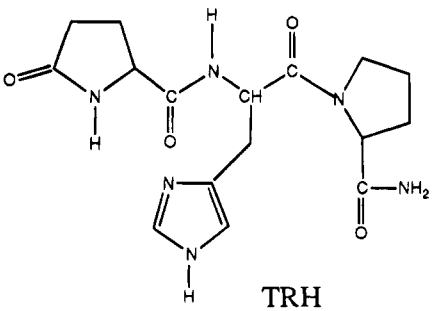
ABSTRACT: To test the hypothesis that pGlu of the thyrotropin-releasing hormone (TRH, pGlu-His-ProNH$_2$) binds to Asn289 in the third extracellular loop (EL3) of its receptor through a hydrogen bonding interaction, we converted Asn289 to Asp (N289D mutant) and measured the potencies of TRH and Pro^{1}TRH for the wild-type and mutant receptors. TRH was 100 times less potent for the N289D receptor than for the wild-type. In contrast, Pro^{1}TRH, which has a protonated proline in place of the pGlu of TRH, was 10 times more potent for the N289D receptor than for the wild-type. A similar result was obtained when Asn289 was converted to Glu, while the potency of Pro^{1}TRH did not change when Asn289 was converted to Ala, confirming that the increased potency of Pro^{1}TRH for the N289D receptor was due to a charge interaction between Pro^{1}TRH and the mutant receptor. These findings are inconsistent with a previous model indicating a direct interaction of the pGlu of TRH with Asn110 in the third transmembrane helix of the receptor (Perlman et al. (1994) *J. Biol. Chem. 269*, 23383−23386). When Asn110 was converted to Asp (N110D mutant), unlike the N289D receptor, the potency of Pro^{1}TRH for the N110D receptor was decreased by >10-fold rather than increased. Therefore, a direct interaction of Asn110 with the pGlu of TRH could not be supported by our experiments. We propose a new model in which the pGlu of TRH binds to Asn289 in EL3 and conclude that, unlike catecholamines which bind completely within the transmembrane domain of their receptors, this tripeptide binds, at least in part, to the extracellular domain of its receptor.

Thyrotropin-releasing hormone (TRH)[1] is a tripeptide (pyroglutamyl-histidyl-prolylamide, pGlu-His-ProNH$_2$, Figure 1), which is released from the hypothalamus, and which stimulates both the secretion and synthesis of thyroid-stimulating hormone and prolactin in the anterior pituitary gland (Wilson & Foster, 1985). In rat pituitary GH$_4$C$_1$ cells, a somatomammotropic cell strain widely used to study TRH actions, the tripeptide enhances the secretion and synthesis of prolactin and growth hormone (Hinkle & Tashjian, 1973; Tashjian, 1979). The action of TRH is mediated by binding to cell surface receptors which activate phospholipase C (Martin, 1983) via a $G_{q/11}$ protein (Aragay et al., 1992; Hsieh & Martin, 1992; Brady et al., 1994) to elevate the concentration of cytosolic free calcium (Albert & Tashjian, 1984). The TRH receptor belongs to the 7 transmembrane receptor superfamily (Straub et al., 1990; Zhao et al., 1992). The goal of this investigation was to identify ligand binding sites in the TRH receptor.



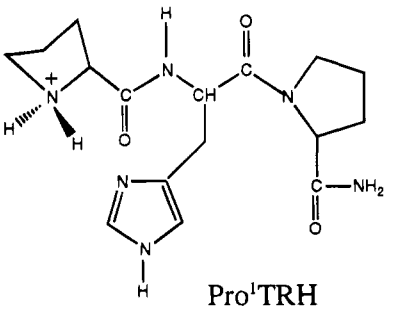


FIGURE 1: Structures of TRH and Pro^{1}TRH. In Pro^{1}TRH, the lactam oxygen on the pyroglutamyl ring of TRH is missing. As a result, ring pucker and a positive charge on the nitrogen are introduced in the five-membered ring of Pro^{1}TRH. In addition, the electron orbital structure of the ring nitrogen changes from planar to tetrahedral. While the lactam moiety including the NH in the pyroglutamyl ring of TRH is planar, neither of the hydrogen atoms in Pro^{1}TRH is coplanar with the ring.

Site-directed mutagenesis has been commonly used to identify ligand binding sites in other G protein-coupled

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[1] Abbreviations: TRH, thyrotropin-releasing hormone (pGlu-His-ProNH$_2$); MeTRH, pGlu-N$^\tau$-MeHis-ProNH$_2$; Hgl^{1}TRH, α-hydroxyglutaryl-γ-lactone-His-ProNH$_2$; Pro^{1}TRH, Pro-His-ProNH$_2$; WT, wild-type; PBS, phosphate buffered saline solution; EL, extracellular loop; TM, transmembrane helix.

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seven transmembrane receptors. Mutation of a residue that makes a direct contact with the ligand is accompanied by a large decrease in the affinity for the ligand, which is commonly called a loss-of-function mutation. But, unequivocal interpretation of the experimental data from loss-of-function mutations is often difficult because of potential indirect effects of the mutation such as a decrease in receptor expression or changes in overall receptor structure. More convincing evidence for direct contact between a specific residue in the receptor and the ligand comes from gain-of-function mutations or complementary mutations in which the altered receptor binds an appropriately modified ligand with a higher affinity than does the wild-type (WT) receptor due to complementation between the modification in the ligand and the mutation in the receptor.

In the β-adrenergic receptor (β-AR) system, a charge interaction between the protonated amine of the ligand and Asp113 in transmembrane helix 3 (TM3) in the receptor has been implied from the large decrease in the affinity of ligands for a mutant receptor in which Asp113 was converted to Asn (Strader et al., 1988). Direct evidence for interaction between the protonated amine and Asp113 came from complementary modifications between the ligand and the receptor; the proposed charge interaction could be replaced by a hydrogen bonding interaction (Strader et al., 1991). Conversion of Asp113 to Ser resulted in a 10 000-fold decrease in the affinity for agonists containing a protonated amine, but this mutant receptor could be fully activated by ligands containing an ester or a ketone in place of the protonated amine. The modified agonists did not activate the WT β-AR.

The lutropin (LH) receptor consists of a particularly long extracellular N-terminal half and a C-terminal half that contains all the 7 transmembrane helices and connecting loops. The high-affinity binding site for the glycoprotein hormone LH is known to reside within the N-terminal half of the receptor. Once bound to the N-terminus, the hormone is proposed to make a secondary contact with the C-terminal half to activate the receptor (Ji & Ji, 1991a,b). Contact of Lys91 in the α-subunit of LH (α-Lys91) with Asp397 in the first extracellular loop of the receptor was identified by complementary modifications of the ligand−receptor pair. When α-Lys91 of the hormone was converted to Asp or the receptor Asp397 was converted to Lys, changes at each of these positions alone resulted in a significant or complete loss of receptor activation. However, when the altered receptor and the altered hormone were combined, activation of the receptor was restored (Ji et al., 1993).

In the gonadotropin-releasing hormone (GnRH) receptor system, an electrostatic interaction between Arg8 of GnRH and Glu301 in the receptor was demonstrated by complementary modifications (Flanagan et al., 1994). When Glu301 of the receptor was converted to Gln, the affinity of GnRH (Arg at position 8) or Lys^{8}GnRH (Lys at position 8) was decreased by 56- and 3-fold, respectively, while that of Glu^8-GnRH (Glu at position 8) was increased by 10-fold.

It is noteworthy that in none of the above examples was the complementation perfect. The binding affinity or receptor activation potency of the appropriately modified pair of ligand and receptor was higher than when only one member of the pair was modified, but the result was always less than that of the WT (or native) pair. This result is probably due to structural changes introduced in either the ligand or the receptor by the modifications which cannot be completely overcome by the complementary interaction. Without detailed knowledge of the three-dimensional structure of the ligand−receptor complex, it is difficult to duplicate an evolutionarily chosen interaction. Nevertheless, complementary modifications in ligand and receptor pairs with gain-of-function will continue to be useful in probing the molecular details of the ligand−receptor interaction.

To identify the ligand binding sites in the TRH receptor, we employed the complementary modification approach. Initial mutagenesis studies on the role of the extracellular sequences in the TRH receptor for ligand binding suggested that Asn289 in the third extracellular loop was important for high affinity TRH binding (Han & Tashjian, 1995). To test the hypothesis that Asn289 binds the lactam moiety of the pGlu ring of TRH, we converted Asn289 to aspartic acid (N289D mutant), which will be negatively charged at neutral pH, and measured receptor activation by TRH and Pro^{1}TRH. In Pro^{1}TRH, the pGlu ring of TRH is replaced with proline, and as a result, the N-terminal ring of the ligand has a positive charge. We predicted that, due to an attractive charge interaction between Pro^{1}TRH and Asp289, the affinity of Pro^{1}TRH would be increased for the N289D receptor. We measured the potencies of TRH and Pro^{1}TRH for stimulation of the wild-type and N289D receptors and present strong evidence to support this model.

Perlman et al. (1994a,b) previously proposed a model for the TRH binding pocket which places TRH entirely within the transmembrane helices, analogous to the binding of catecholamines to their receptors. This conclusion was based on evidence suggesting a direct interaction of Tyr106 in the putative third transmembrane helix (TM3) of the receptor with the carbonyl oxygen of the pyroglutamyl ring of TRH (Perlman et al., 1994b) and a direct interaction of Asn110 in TM3 with the NH group of the pyroglutamyl ring (Perlman et al., 1994a). Because, in our model, TRH binds to its receptor in essentially the opposite orientation to that proposed by Perlman et al., we also tested their model by converting Asn110 to aspartic acid and measured the potencies of TRH and Pro^{1}TRH for this mutant; the results obtained do not support their model.

MATERIALS AND METHODS

Materials. Cell culture plasticware was obtained from Falcon Labware (Division of Becton Dickinson, Lincoln Park, NJ). Culture medium and sera were purchased from GIBCO Laboratories (Grand Island, NY). TRH, MeTRH, and Pro^{1}TRH were purchased from Bachem California (Torrance, CA). [^{3}H]MeTRH (56−64 Ci/mmol) was purchased from DuPont NEN (Wilmington, DE). Fura-2 AM was obtained from Molecular Probes (Eugene, OR). Other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

Mutagenesis of the Receptor. The *Hin*dIII−*Xmn*I fragment of the cDNA containing the entire coding region of the rat TRH receptor (Zhao et al., 1992) was subcloned into the pCDM8 vector (Invitrogen, San Diego, CA) between the *Hin*dIII and *Not*I sites using a *Xmn*I/*Not*I linker. Mutagenesis of the receptor was performed by Kunkel's method (Kunkel, 1985) using the Mutagen kit (Bio-Rad, Hercules, CA). Mutagenic oligos often contained an additonal silent mutation to create a restriction site which was used for screening of

the mutants. All mutant receptors were confirmed by sequencing the mutant plasmid using the dideoxy chain termination method (Sanger et al., 1977). Plasmid DNA was purified with a QIAGEN kit (QIAGEN Inc., Chatsworth, CA) before sequencing and transfection into $GH_1 2C_1 b$ cells.

Microinjection of mRNA into Xenopus Oocytes. Oocytes were surgically removed from adult female *Xenopus laevis* (Nasco, Fort Atkinson, WI) after anesthetizing with 0.2% 3-aminobenzoic acid ethyl ester (Sigma, St. Louis). Stage V oocytes were selected, and the follicular membranes were removed manually under a dissecting microscope. mRNA for TRH receptors was obtained by in vitro transcription of linearized plasmids containing the cDNA coding for the receptor using the mMESSAGE mMACHINE kit (Ambion, Austin, TX) according to the manufacturer's instructions. The mRNA was dissolved in water at a concentration of 0.5 ng/nL. About 15 ng of mRNA was injected into each oocyte. Oocytes were kept in ND96 solution (96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES, pH 7.5) during the injection. Injected oocytes were incubated in L-15 medium (70% Leibovitz's L-15 medium, 10 mM HEPES, pH 7.5) supplemented with 100 units/mL penicillin and 100 μg/mL streptomycin at 17−20 °C for 36 h before electrophysiological measurement. In some cases, the oocytes were treated briefly (<1 h) with collagenase (2 mg/mL, type IA, Sigma) before manual removal of the follicular membranes. Collagenase treatment was performed in calcium-free OR2 solution (82.5 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 5 mM HEPES, pH 7.5). Collagenase-treated oocytes were washed thoroughly with calcium-free OR2 solution before transfer to calcium-containing ND96 solution. Collagenase treatment significantly reduced both the viability of the oocytes and the magnitude of the response to a given concentration of an agonist. Collagenase-treated oocytes showed a 10-fold right shift in the dose−response relationship to agonists as compared to oocytes from the same donor not treated with collagenase. This finding was not due to degradation of endogenous TRH receptors in the oocytes because uninjected oocytes did not respond to TRH (up to 10 μM) either before or after collagenase treatment. Similar observations were made when mRNA for luciferase was injected; luciferase activity was 10 times higher in oocytes not treated with collagenase (J. Gutierrez and B. Han, unpublished observations).

Electrophysiology. Whole-cell current measurements in *Xenopus* oocytes were performed using the two-electrode voltage-clamp method (Zhao et al., 1992) in a chamber continuously perfused with ND96 solution at 2 mL/min. Agonists were freshly prepared at working concentrations in ND96 and applied for 1 min in place of the control perfusion solution. The resting membrane potential of the oocytes ranged between −35 and −50 mV. Membrane potential was clamped at −60 mV. Membrane currents were stored on a Recorder 220 (Gould Inc., Valley View, OH). Under the conditions used, a response <100 nA was difficult to distinguish from background. Peak current was obtained from measurement of the peak height on the calibrated graphical output. Graphical outputs were scanned into a computer using a hand-held scanner at 300 dpi resolution for storage and presentation of the results. The scanned image was indistinguishable from the original. For presentation, the scanned images were labeled using Corel Draw (Corel Corp., Ottawa, Ontario, Canada) and printed without processing of the image. In some experiments, the same oocytes were exposed to two successive agonists. Treatment of oocytes with one agonist may result in desensitization to the second agonist. The extent of desensitization depended on several factors, including the magnitude of the response to the first agonist, duration of washing between the two treatments, and the relative concentration (or potency) of the two agonists. When the response to the first agonist was small and the second agonist was more potent (>100-fold) than the first, the response to the second agonist could be measured without attenuation induced by the first agonist if sufficient washing was allowed between the treatments. But, if the first agonist was more potent than the second, a response to the second agonist could not be measured. When the potencies of the two agonists were similar, the response to the second agonist was reduced, leading to underestimation of the second response.

Cell Culture and Transient Expression of TRH Receptors in $GH_1 2C_1 b$ Cells. Wild type and mutant TRH receptors were transiently expressed in $GH_1 2C_1 b$ cells for ligand binding and signal transduction assays. $GH_1 2C_1 b$ cells, a subclone of $GH_1 2C_1$ cells (Tashjian, 1979), are rat pituitary cells which lack TRH receptor expression and, therefore, serve as an ideal null cell line for expression of the receptor. $GH_1 2C_1 b$ cells were cultured as described (Tashjian, 1979). Briefly, cells were fed every 3−4 days with Ham's F-10 medium supplemented with 15% horse serum and 2.5% fetal bovine serum (F10+ medium). Cultures were maintained at 37 °C in a humidified atmosphere of 5% CO_2/95% air. Exponentially growing $GH_1 2C_1 b$ cells were split 1:2−1:5 and seeded into 100 mm dishes 1 day before transfection. The transfecting DNA was prepared by mixing phosphate buffered saline (PBS) containing an appropriate amount (see below) of DNA with an equal volume of DEAE-dextran (1 mg/mL) dissolved in PBS. Cells were washed twice with PBS, and 340 μL of the DNA mixture was applied dropwise to each 100 mm dish and gently swirled. After 15 min at room temperature (20 °C), the DNA solution was aspirated, and the cells were incubated with 5 mL of 10% DMSO in PBS for 2 min. Cells were washed once with 5 mL of PBS and incubated for 2−3 h at 37 °C with 5 mL of F10+ medium containing 100 μM chloroquine. The chloroquine-containing medium was then removed, and the cells were washed once with PBS and incubated with 8 mL of F10+ medium. Typical transfection efficiency was 2% of the total cell population as measured by confocal microscopy using a viral hemagglutinin A (HA)-tagged TRH receptor and a monoclonal antibody to the HA tag (C. Petrou and A. H. Tashjian, Jr., unpublished data).

Measurement of $[Ca^{2+}]_i$. Measurement of cytosolic free calcium concentration was performed using Fura-2 as the indicator dye. Ninety-six hours after transfection, cells were detached from the dishes by incubation with HBSS solution (118 mM NaCl, 4.6 mM KCl, 10 mM D-glucose, 20 mM Hepes, pH 7.4) containing 1 mM EDTA, harvested, and washed once with warm HBSS solution containing 1 mM $CaCl_2$ (HBSS/Ca) and incubated with Fura-2 AM (5 μg of Fura-2 AM for 10^6 cells in 5 mL of HBSS/Ca) for 45 min at 37 °C with continuous swirling. Cells were washed twice and resuspended in 3 mL of HBSS/Ca, and measurement of fluorescence was performed in a Spex Fluorolog F111A spectrofluorometer (Spex Industries, Edison, NJ) at an excitation wavelength = 342 nm and an emission wavelength

= 492 nm. $[Ca^{2+}]_i$ was calibrated using the formula $[Ca^{2+}]_i = K_d(F - F_{min})/(F_{max} - F)$, where K_d was 224 nM for Fura-2, F was the fluorescence signal in arbitrary units, F_{max} was the fluorescence obtained after permeabilization of cells with digitonin in the presence of the 2 mM $CaCl_2$, and F_{min} was the fluorescence obtained by chelating the Ca^{2+} with 5 mM EGTA. Basal $[Ca^{2+}]_i$ was 100−150 nM and increased 2- to 3-fold on agonist addition. Because transfection efficiency was <5%, measured $[Ca^{2+}]_i$ after agonist addition in the total population of cells does not represent the true change in $[Ca^{2+}]_i$ in the subpopulation of responding cells. Therefore, we show the uncalibrated fluorescence change in arbitrary units.

RESULTS

Structures of TRH and Pro^{1}TRH. Pro^{1}TRH differs from TRH in that the carbonyl oxygen on the pyroglutamyl ring of TRH is missing (Figure 1). This modification eliminates a potential hydrogen bonding interaction of the ligand with the receptor through the carbonyl oxygen. In addition, pucker in the N-terminal five-membered ring of Pro^{1}TRH will be significantly greater than that in the pyroglutamyl ring of TRH, because the double bond character between the carbonyl carbon and ring nitrogen of TRH is lost in Pro^1-TRH (Stryer, 1988). More importantly, a positive charge will be introduced on the ring nitrogen of Pro^{1}TRH at neutral pH due to the nature of the secondary amine. The pK_a of the ring nitrogen in proline is 10.6 (Streitwieser & Heathcock, 1981b). The charge on the ring nitrogen of Pro^{1}TRH can potentially interfere with binding to the WT receptor. Furthermore, a possible hydrogen bonding interaction with the ring NH of TRH may also be impaired in Pro^{1}TRH, because neither of the two hydrogen atoms attached to the ring nitrogen in Pro^{1}TRH will be on the same plane with the ring. This conclusion follows because, while the electron orbital of the ring nitrogen in TRH is planar due to the double bond character of the C−N bond (Stryer, 1988), the orbital structure of the ring nitrogen in Pro^{1}TRH will be sp^3, which is tetrahedral (Streitwieser & Heathcock, 1981a). Thus, the proper geometry of the NH for optimal hydrogen bond formation with the receptor may be lost in Pro^{1}TRH. From these considerations in aggregate, a substantial decrease in the affinity of Pro^{1}TRH for the WT TRH receptor can be expected. Indeed, the reported K_d of Pro^{1}TRH is 560 μM, which is 43 000-fold higher than the K_d of 13 nM for TRH (Perlman et al., 1994b).

Increased Potency of Pro^{1}TRH for the N289D Receptor. The model of a direct interaction of Asn289 with the pyroglutamyl ring NH of TRH predicted that the affinity of Pro^{1}TRH, which has a positive charge on the N-terminal ring nitrogen, would be increased, because of a favorable charge interaction, if Asn289 were replaced by an acidic residue. To test this possibility, we converted Asn289 to aspartic acid (N289D mutant). The N289D mutant showed no specific binding of 4 nM [^{3}H]MeTRH, when transiently expressed in GH_12C_1b cells (data not shown), consistent with a large (>100-fold) decrease in the affinity for MeTRH. Because binding affinities of TRH and Pro^{1}TRH could not be determined directly for the N289D receptor, we measured the potencies of these agonists for the WT and N289D receptors and used these data to estimate the relative affinities of the receptors for the ligands.

The potencies of TRH and various TRH analogs show a strong linear relationship with their affinities for the receptor over a wide range (>10 000-fold) (Hinkle et al., 1974). For the WT receptor, the affinity of TRH was 43 000 times higher than that of Pro^{1}TRH, while the potency of TRH for stimulation of inositol polyphosphate formation was 110 000 times higher than that of Pro^{1}TRH (Perlman et al., 1994b). The difference between the two estimates was less than 3-fold. As will be shown below, the measured difference in the potencies of TRH and Pro^{1}TRH for stimulation of the chloride current in *Xenopus* oocytes expressing the WT receptor also correlated well with the difference in the affinities between the two ligands. Furthermore, Pro^{1}TRH, at sufficiently high concentrations, shows the same full agonist activity as TRH, and the dose−response curve for Pro^{1}TRH is parallel to that of TRH (Perlman et al., 1994b). These findings justify estimation of relative affinities of TRH and Pro^{1}TRH for TRH receptors using measurements of agonist potencies of these ligands (Limbird, 1986). To measure receptor activation by the agonists, the receptors were expressed in *Xenopus* oocytes, and the intracellular calcium-induced chloride current elicited by TRH and Pro^1-TRH was measured (Zhao et al., 1992).

Maximum chloride currents from oocytes expressing either the WT or the N289D receptor elicited by 10 μM TRH were similar (2−3 μA in collagenase-treated oocytes and 4−5 μA in untreated oocytes, see Materials and Methods). Therefore, the level of receptor expression did not appear to be significantly different between cells expressing WT and N289D receptors. However, since the magnitude of the chloride current and the potency of agonists can be affected by the level of receptor expression and variability between individual oocytes, as well as the intrinsic properties of the receptor, we took additional care to eliminate the effect of potential differences in receptor expression and variability between oocytes. For this purpose, we compared responses to TRH and Pro^{1}TRH in the same oocytes to estimate the relative potencies of the two agonists. After exposure to the first agonist, the oocyte was washed for 10 min and the second agonist was applied. Because treatment with the first agonist can desensitize the receptor (or the oocyte), the response to the second agonist treatment will be underestimated. Therefore, if the magnitude of response to the second agonist is smaller than the response to the first, the relative potency of the two agonists cannot be estimated. On the other hand, if the magnitude of the response to the second agonist is equal to or larger than that of the first agonist, it can be concluded that the second agonist was at least as potent as the first. Such internal comparisons are not affected by the level of receptor expression or variability between oocytes and, thus, ensure measurement of the intrinsic properties of the agonist−receptor interaction.

In oocytes expressing the WT receptor, 1 μM Pro^{1}TRH elicited no response (n = 5) while the same oocytes, after washing for 10 min, reproducibly gave a large response to 1 nM TRH (Figure 2A). Because the response to 1 nM TRH was always (n > 5) larger than the response to 10 μM Pro^1-TRH in the same oocyte (Figure 2B), we estimated that the potency of TRH for the WT receptor was >10 000 times higher than that of Pro^{1}TRH. Reversing the order of application of the two agonists did not affect this conclusion (data not shown). This estimation is consistent with the

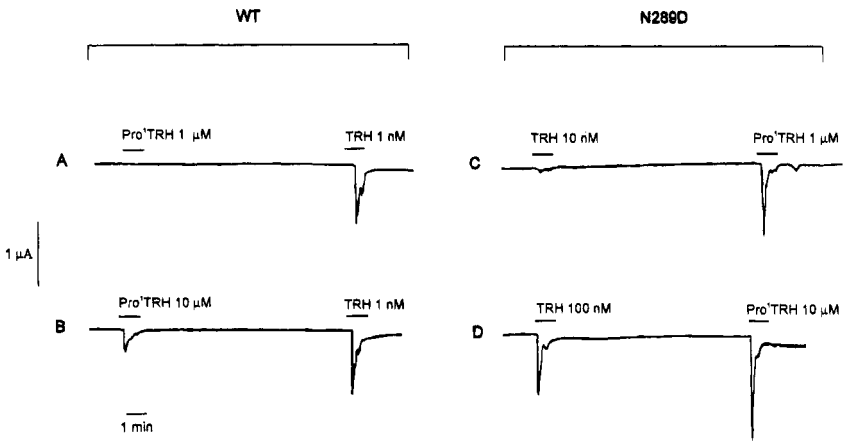

FIGURE 2: Increased potency of Pro¹TRH for the N289D receptor. The same oocytes expressing either WT or N289D receptors were exposed successively to different concentrations of TRH or Pro¹TRH, and the magnitude of the chloride current was measured to compare the relative potency of the two agonists for each type of receptor. Oocytes were washed for 10 min before the second agonist was applied. The potency of Pro¹TRH was increased while that of TRH was decreased for the N289D receptor. Each experiment was repeated 4 or more times from the same batch of oocytes. In addition, at least 4 different batches of oocytes were tested, and each batch gave similar results. Uninjected oocytes gave no response to 10 μM TRH or Pro¹TRH. Stage V or VI oocytes were defoliculated manually after treatment with collagenase for 45 min. Injection of RNA and electrophysiological measurements were performed as described in Materials and Methods.

43 000-fold lower K_d of TRH for the WT receptor than the K_d of Pro¹TRH (Perlman et al., 1994b) and confirms that data obtained from oocytes can be extrapolated to TRH receptors in mammalian cells.

As was predicted from the model of a direct interaction of Asn289 with the lactam moiety of the pGlu ring of TRH, the potency of Pro¹TRH was increased for the N289D receptor. In oocytes expressing the N289D receptor, 1 μM Pro¹TRH gave a reproducible response in 9 out of 9 experiments (Figure 2C), a >10-fold increase in the potency of Pro¹TRH for the N289D receptor as compared to the WT receptor. This result was in contrast to the marked decrease in the potency of TRH for the N289D receptor. In oocytes expressing the N289D receptor, a reproducible response to 10 nM TRH was not measurable, while the same oocytes, after washing, clearly responded to 1 μM Pro¹TRH (Figure 2C), demonstrating that the lack of response to 10 nM TRH was due to the decreased potency of TRH for the N289D receptor, and not due to decreased receptor expression or variability between oocytes. Reproducible responses were obtained with 100 nM TRH, a 100-fold decrease in the potency of TRH, but this response was always smaller than the response to 10 μM Pro¹TRH in the same oocytes (n = 6, Figure 2D). These results demonstrate that the potency of Pro¹TRH was increased for the N289D receptor, while the potency of TRH was decreased. Because the relative potency of TRH to Pro¹TRH was >10 000-fold for the WT receptor while, for the N289D receptor, it was <100-fold, we estimated that there was about a 1000-fold increase in the relative potency of Pro¹TRH to TRH for the N289D receptor.

Specificity of the Charge Interaction between Pro¹TRH and Asp289 As Determined by Threshold Ligand Concentrations. To test if the increased potency of Pro¹TRH for the N289D receptor was due to the attractive charge interaction, we converted Asn289 to Glu (N289E mutant) and Ala (N289A mutant) and measured the threshold concentrations of TRH and Pro¹TRH to activate each of these mutant receptors. The threshold concentration of an agonist (Figure 3, first and second vertical columns), measured at 10-fold dose intervals, was defined as the lowest concentration of the agonist to yield a reproducible electrophysiological response. The response to a threshold concentration (designated the threshold response) was a current with a rapid rise and fall (width at half-height <30 s), which ranged between 200 nA and 1.5 μA at the peak (depending on the receptor and the health of the oocytes). In order to ensure that the measured threshold agonist concentration was not affected by variability between oocytes, we employed an internal control for each oocyte. At a concentration 10-fold lower than threshold (Figure 3, first treatments in the third and fourth vertical columns), the response was either not detectable or <200 nA with a slow rise and fall (width at half-height >1 min). The same oocyte was then washed for 5 min with buffer and then exposed to the same agonist at a concentration 10-fold higher than threshold (Figure 3, second treatments in the third and fourth vertical columns). A clear response to the second exposure demonstrated that lack of response to the first treatment was not due to an artifact and indicated that the measured threshold concentration was indeed the lowest agonist concentration required to elicit a reproducible response. A series of such experiments is presented in Figure 3 for WT and each of the mutant receptors. Because collagenase treatment of the oocytes lowered the responsiveness of the oocytes and increased the variability between oocytes, we omitted collagenase treatment before defoliculation of the oocytes in these experiments.

In oocytes expressing the WT receptor, threshold responses were obtained at 0.1 nM TRH and 1 μM Pro¹TRH. For the N289D receptor, the threshold concentration of TRH increased to 10 nM, while the threshold concentration of Pro¹TRH decreased to 0.1 μM (Figure 3). These results confirmed the estimation of a 1000-fold increase in the relative potency of Pro¹TRH to TRH for the N289D receptor derived from internal comparisons in single oocytes (Figure 2).

• In oocytes expressing the N289E receptor, in which Asn289 was converted to another acidic residue, glutamic acid, essentially the same result was obtained as with the N289D receptor; the threshold concentration of TRH was increased by a factor of 100, while the threshold concentration of Pro¹TRH was decreased >10-fold (Figure 3). On

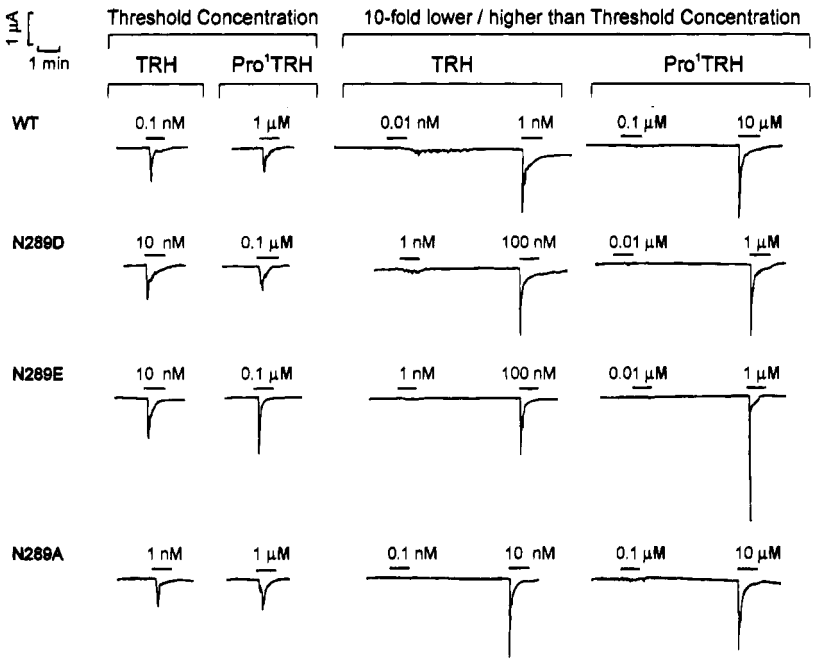

FIGURE 3: Increased potency of Pro¹TRH for the N289D receptor is due to a charge interaction between Pro¹TRH and Asp289. The threshold concentrations of TRH and Pro¹TRH (vertical columns 1 and 2, labeled TRH and Pro¹TRH) were 0.1 nM and 1 μM for WT, 10 nM and 0.1 μM for N289D, 10 nM and 0.1 μM for N289E, and 1 nM and 1 μM for N289A, respectively. The threshold concentration of Pro¹TRH decreased 10-fold when the Asn289 was converted to Asp or Glu (N289D and N289E, respectively) while there was no change when Asn289 was converted to Ala (N289A receptor). Lack of response to 10-fold lower than a threshold concentration was not due to individual variation among oocytes, because the same oocyte responded strongly to an agonist concentration 10-fold higher than threshold (vertical columns 3 and 4, labeled TRH and Pro¹TRH, see text). Data shown were obtained from a single batch of oocytes. Each experiment was repeated 4 or more times using the same batch of oocytes. In addition, at least three different batches of oocytes were tested, and each batch gave similar results. Uninjected oocytes gave no response to 10 μM TRH or Pro¹TRH. Stage V oocytes were defoliculated manually without collagenase treatment.

the other hand, in oocytes expressing the N289A receptor, in which Asn289 was converted to a neutral amino acid, alanine, the threshold concentration of TRH was increased 10-fold, while the threshold concentration of Pro¹TRH was not altered. These results demonstrate the specificity of the interaction between the positive charge on Pro¹TRH and the negative charge on residue 289 to increase the potency of Pro¹TRH for the N289D receptor.

Concentration–Response Relationships for TRH and Pro¹TRH with WT and Mutant TRH Receptors. To confirm that the threshold concentrations of TRH and Pro¹TRH appropriately represented the potency of each agonist, we compared the electrophysiological responses at a range of concentrations of the agonists (Figure 4). For the WT receptor, the potency of TRH was >10 000 times higher than that of Pro¹TRH. For N289D and N289E receptors, the concentration–response plots for Pro¹TRH were shifted to the left by a factor of 10 or more, while the concentration–response plots for TRH were shifted to the right by a factor of 100 (Figure 4A). For the N289A receptor, the concentration–response plot for Pro¹TRH did not change, while the concentration–response plot for TRH was shifted to the right by a factor of 10 (Figure 4B). These results confirm the relative potencies of the two agonists for each TRH receptor estimated from internal comparisons in single oocytes and establish the validity of using threshold agonist concentrations as a means to measure potencies of agonists for TRH receptors.

Charge Interaction of Pro¹TRH Is Specific for Residue 289. In order to test if the charge interaction of Pro¹TRH was specific for the negative charge on residue 289 but not other residues, we converted Ser290, a neighboring amino acid, to aspartic acid (S290D receptor) and measured the potency of TRH and Pro¹TRH. Ser290 was also found to be important for high affinity TRH binding. Conversion of Ser290 to either Ala or Asp resulted in a large decrease in receptor affinity for MeTRH (data not shown). S290D receptor showed a large decrease in the potency for TRH (Figure 5). Unlike the N289D receptor, however, there was no increase in potency for Pro¹TRH with the S290D receptor. In fact, the potency of Pro¹TRH was decreased to an extent similar to that for TRH (Figure 5). These results demonstrate that the charge interaction between Pro¹TRH and Asp289 in the N289D receptor is not a long-range interaction.

pGlu of TRH Does Not Bind to Asn110 in the Third Transmembrane Helix of the Receptor. Because Perlman et al. (1994a) proposed that the pyroglutamyl ring NH of TRH interacts directly with Asn110 in TM3, we examined their conclusion by replacing Asn110 with Asp (N110D mutant) and measured the potencies of TRH and Pro¹TRH. In oocytes expressing the WT receptor, 0.1 nM TRH (Figure 6A) or 1 μM Pro¹TRH (Figure 6B) elicited small reproducible responses, consistent with the threshold concentrations of these agonists for the WT receptor (Figure 3). In oocytes expressing the N110D receptor, 0.1 nM TRH again elicited a reproducible response (Figure 6D), indicating that potency of TRH for N110D receptor was not significantly reduced. Examination of the full dose–response relationship confirmed that maximal stimulation of the N110D receptor was comparable to that of the WT receptor and that the decrease in the potency of TRH was <10-fold (Figure 7). On the other hand, 10 μM Pro¹TRH elicited no response ($n = 5$) in the N110D receptor, while the same oocyte reproducibly gave a strong response to 1 nM TRH (Figure 6E), demon-

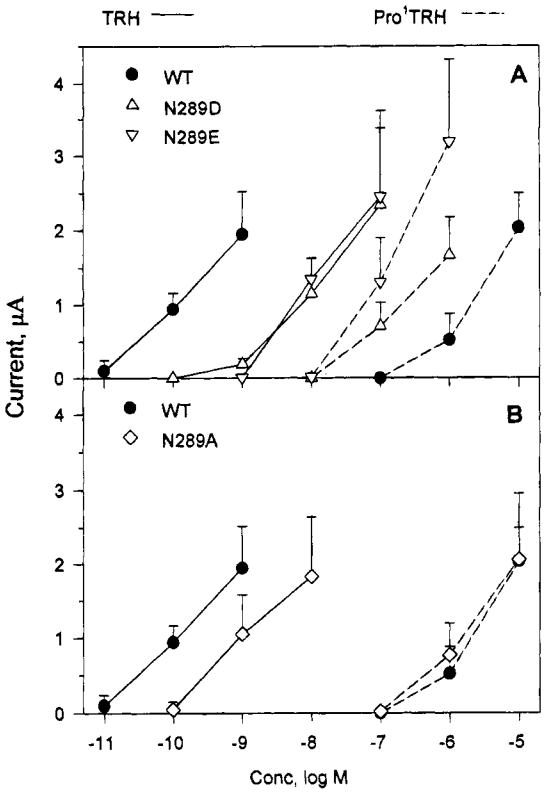

FIGURE 4: Concentration-response relationships for TRH and Pro^1-TRH with WT and mutant TRH receptors. TRH (solid lines)- or Pro^{1}TRH (dashed lines)-induced peak chloride currents were measured in oocytes expressing each receptor. (A) Conversion of Asn289 to negatively charged residues, Asp (N289D receptor) or Glu (N289E receptor), resulted in a 100-fold decrease in the potency of TRH and a >10-fold increase in the potency of Pro^{1}TRH. (B) Conversion of Asn289 to a neutral residue, Ala (N289A receptor), resulted in a 10-fold decrease in the potency of TRH, but no change in the potency of Pro^{1}TRH. Stage V oocytes were defoliculated manually without collagenase treatment. Each data point is an average of the peak current from 3−8 different oocytes, and the brackets give the SD.

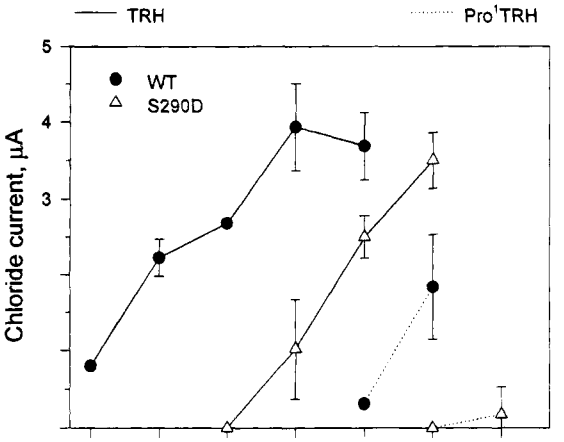

FIGURE 5: Charge interaction of Pro^{1}TRH is specific for residue 289 of the receptor. Ser290, a neighboring amino acid to Asn289, was converted to aspartic acid (S290D receptor). TRH (solid lines)- and Pro^{1}TRH (dashed lines)-induced peak chloride currents were measured in oocytes expressing the WT or the S290D receptor. Potencies of both TRH and Pro^{1}TRH were decreased for the S290D receptor. Stage V oocytes were defoliculated manually without collagenase treatment. Similar results were obtained using two additional batches of oocytes. Each data point is an average of peak current from 3−5 different oocytes, and the brackets give the SD.

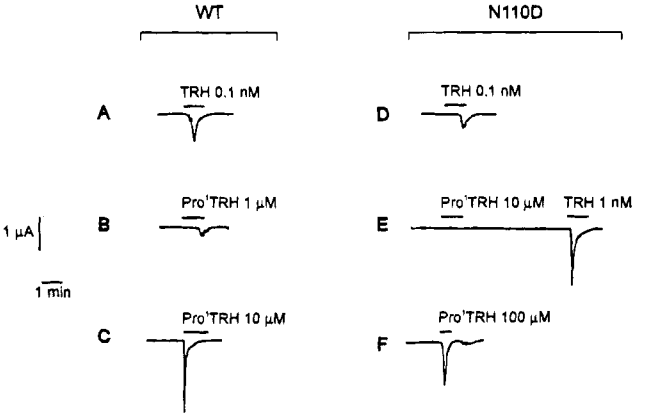

FIGURE 6: pGlu of TRH does not bind Asn110. Threshold concentrations of TRH and Pro^{1}TRH were measured to test whether pGlu of TRH binds to Asn110 as proposed by Perlman et al. (1994a). In oocytes expressing the WT receptor (A, B, and C), the threshold concentrations of TRH (A) and Pro^{1}TRH (B) were 0.1 nM and 1 μM, respectively. Pro^{1}TRH at 10 μM gave a strong response (C). In oocytes expressing the N110D receptor (D, E, and F), the threshold concentration of TRH did not change significantly (D), while that of Pro^{1}TRH increased 100-fold (E and F). Pro^1-TRH at 10 μM failed to elicit a response (E), while the same oocyte responded strongly to 1 nM TRH. Pro^{1}TRH gave a threshold response at 100 μM concentration (F). Stage V oocytes were defoliculated manually without collagenase treatment. Data shown are representative of 3 or more identical experiments, each performed with the same batch of oocytes. Similar results were obtained using two additional batches of oocytes. Two independent clones of the N110D receptor were tested and gave identical results.

strating that the lack of response to 10 μM Pro^{1}TRH was indeed due to the low potency of Pro^{1}TRH. Pro^{1}TRH (100 μM) reproducibly gave a significant response in oocytes expressing the N110D receptor (n = 3, Figure 6F), but the magnitude of the response was smaller than the response to 10 μM Pro^{1}TRH in oocytes expressing the WT receptor (n = 4, Figures 6C and 7). From the results of these experiments, we estimate that the potency of Pro^{1}TRH for the N110D receptor was decreased >10-fold. The decrease in potency of Pro^{1}TRH for the N110D receptor does not support the proposed direct interaction of Asn110 with the pyroglutamyl ring NH of TRH (Perlman et al., 1994a).

Increased Potency of Pro^{1}TRH for the N289D Receptor As Measured by Changes in $[Ca^{2+}]_i$ in GH_1C_1b Cells Transiently Expressing WT or N289D Receptors. To demonstrate that the findings presented above in *Xenopus* oocytes were not unique to the heterologous expression system, we measured cytosolic calcium responses in GH_1C_1b cells transiently expressing WT or N289D receptors (Figure 8, left and right, respectively). Because GH_1C_1b cells lack TRH receptors but are derived from the same rat pituitary parental cell strain as GH_3 and GH_4C_1 cells, the mutant receptor can be compared to the WT receptor in its native cellular environment. However, because the efficiency of transfection is low (<5% of the cells in the total population), the response to agonists is small. Consistent with the low affinity of Pro^{1}TRH for the WT receptor, 100 μM Pro^{1}TRH did not give a reproducible acute change in $[Ca^{2+}]_i$ in cells expressing the WT receptor (Figure 8 A,B). However, the same cells clearly responded to 10 μM MeTRH, demonstrating that the lack of response to 100 μM Pro^{1}TRH was due to the low affinity of Pro^{1}TRH for the WT receptor and that 100 μM Pro^{1}TRH was not sufficient to desensitize the receptor. In cells expressing the N289D receptor, 10 μM MeTRH elicited a clear response (Figure 8C). Consistent

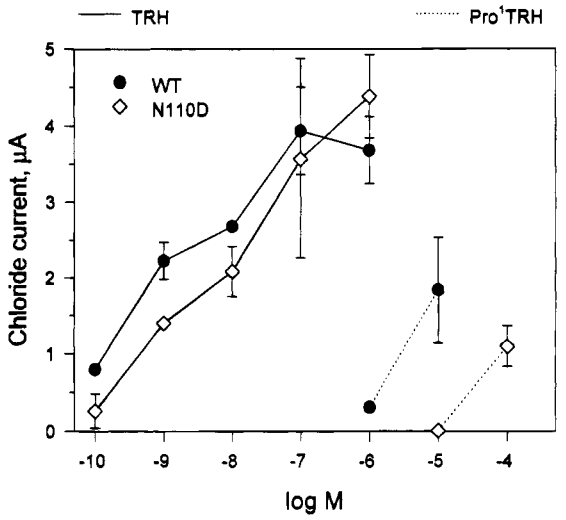

FIGURE 7: Concentration−response relationships for TRH and Pro¹-TRH with WT and N110D receptors. TRH (solid lines)- or Pro¹-TRH (dashed lines)-induced peak chloride currents were measured in oocytes expressing the WT or the N110D receptor. Potency of TRH for the N110D receptor decreased <10-fold, while that of Pro¹TRH decreased >10-fold. Oocytes were not treated with collagenase before defoliculation. Each data point is an average of the peak current from 2−4 different oocytes from the same batch, and the brackets give the SD.

with the increased potency of Pro¹TRH for the N289D receptor, 100 μM Pro¹TRH gave a reproducible acute rise in $[Ca^{2+}]_i$ in cells expressing the N289D receptor (Figure 8D,E). Subsequent exposure of the same cells to 100 μM MeTRH elicited no further response (Figure 8D,E), indicating that 100 μM Pro¹TRH had desensitized signaling via the N289D receptor. These results confirm the conclusions drawn from the oocyte expression system, namely, that the potency of Pro¹TRH was increased for the N289D receptor.

DISCUSSION

From the data presented in this paper, we conclude that there is a direct interaction between the pyroglutamyl ring of TRH and Asn289 in the TRH receptor. This conclusion is based on the observation that the potency of Pro¹TRH, which has a positive charge on the ring nitrogen (Figure 1), was increased for the N289D receptor, in which Asn289 was converted to a negatively charged residue, Asp. From the model of direct interaction between the pyroglutamyl ring NH of TRH and Asn289 in the receptor, we predicted that the affinity of Pro¹TRH should be enhanced for the N289D receptor. Because binding of [³H]MeTRH to the N289D receptor was low, it was not feasible to measure directly the binding affinities of TRH and Pro¹TRH for this receptor. Therefore, we measured the potencies of TRH and Pro¹TRH for WT and N289D receptors to test the prediction that the affinity of Pro¹TRH was increased for the N289D receptor. The validity of this approach was discussed in the Results. Although the potencies of agonists for receptor activation can be affected by the level of receptor expression, and although we could not measure directly expression of the N289D receptor in oocytes or in GH_1C_1b cells, this limitation should not affect the conclusions drawn from the experiments performed which included the use of internal comparisons. We compared the relative potencies of TRH and Pro¹TRH for the same TRH receptor in the same oocyte and, thereby, eliminated the effect of differences in the receptor expression. The reproducibility of such experiments with internal controls performed in many oocytes derived

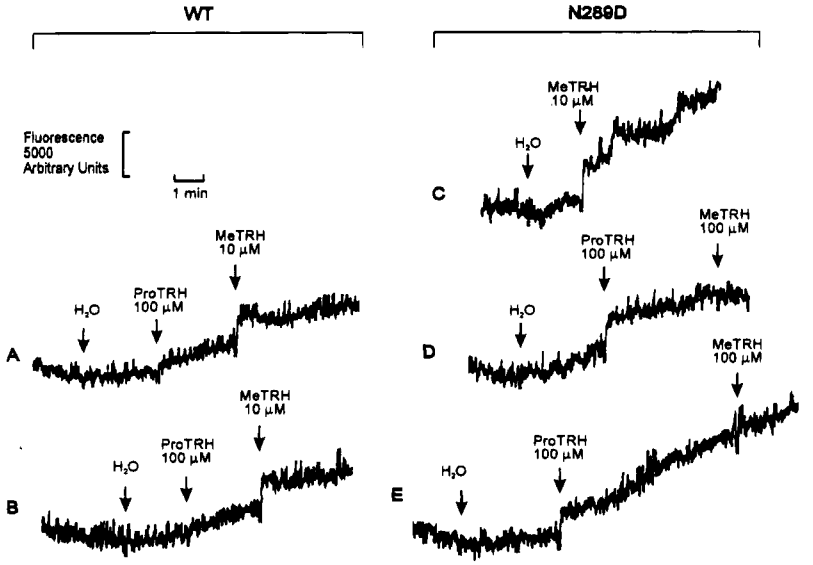

FIGURE 8: Increased potency of Pro¹TRH for the N289D receptor as measured by changes in $[Ca^{2+}]_i$ in GH_1C_1b cells transiently expressing WT or N289D receptors. Ca^{2+} signaling was measured to confirm the increased potency of Pro¹TRH for the N289D receptor in rat pituitary GH_1C_1b cells. 100 μM Pro¹TRH did not elicit Ca^{2+} response in cells expressing the WT receptor, while the same cells responded clearly to subsequent addition of 10 μM MeTRH (A and B). In cells expressing the N289D receptor (C, D, and E), 100 μM Pro¹TRH elicited a clear response (D and E), confirming increased potency of Pro¹TRH for the N289D receptor. Subsequent exposure of the same cells to 100 μM MeTRH failed to give additional response (D and E), while 10 μM MeTRH elicited a clear response when applied without prior exposure to 100 μM Pro¹TRH (C). Cells transfected with each receptor DNA were loaded with Fura-2 AM, and changes in $[Ca^{2+}]_i$ were deduced from changes in the fluorescence signal (see Materials and Methods). Data presented were obtained from a single large batch of cells divided into two groups and transfected in parallel. Cells transfected with a single receptor cDNA (WT or N289D) were pooled and loaded with Fura-2 AM, and then divided for testing just before fluorescence measurements. MeTRH (10 μM) did not alter fluorescence in nontransfected GH_1C_1b cells. Additions are shown by the arrows, and fluorescence is given in arbitrary units. The gradual rise in fluorescence in the cells expressing the WT receptor after addition of Pro¹TRH does not represent a true response to agonist; it is merely base-line drift. A true response is characterized by an abrupt, rapid rise in fluorescence to a new base line.

from several different donor frogs, with or without collagenase treatment, validates the approach because it is unlikely that receptor expression was identical in all the oocytes. Changes in receptor expression are likely to affect equally the potencies of both TRH and Pro^{1}TRH and, therefore, are unlikely to affect the relative potency of Pro^{1}TRH to TRH. However, relative potency of Pro^{1}TRH to TRH increased up to 1000-fold for the N289D receptor compared to the WT receptor. The potency of Pro^{1}TRH increased for the N289D receptor while that of TRH decreased. Such changes in the potencies of TRH and Pro^{1}TRH, in opposite directions, cannot be due to changes in receptor expression; they can only be explained by a change in the intrinsic properties of the receptor. Use of internal controls within single oocytes also eliminated potential artifacts in the measurement of threshold agonist concentrations. In these experiments, the lack of response to a subthreshold concentration of agonist was shown to be due to low agonist concentration, rather than variability between oocytes, by measuring a large response to a second application of a higher concentration of the same agonist in the same oocyte.

Using electrophysiological measurements in *Xenopus* oocytes expressing WT or N289D receptors, we found that TRH was at least 10 000 times more potent than Pro^{1}TRH for the WT receptor, consistent with the 43 000 times higher affinity of TRH than Pro^{1}TRH for the WT receptor measured in mammalian cells (Perlman et al., 1994b). As predicted from our model, the potency of Pro^{1}TRH for the N289D receptor was increased by at least 10-fold, while the potency of TRH was decreased 100-fold; the relative potency of Pro^1-TRH to TRH was increased by 1000-fold. Essentially the same result was obtained when Asn289 was converted to another negatively charged amino acid, glutamic acid. On the other hand, when Asn289 was converted to the neutral amino acid, alanine, the potency of Pro^{1}TRH was unaltered while the potency of TRH was decreased, demonstrating the importance of a charge interaction between ligand and receptor to explain the increased potency of Pro^{1}TRH for the N289D receptor. This charge interaction was specific to residue 289 because, when an adjacent residue Ser290 was converted to aspartic acid, both TRH and Pro^{1}TRH showed large (>100-fold) decreases in potency. These experiments provide strong evidence for a direct contact between the N-terminal prolyl ring nitrogen of Pro^{1}TRH and Asp289 of the N289D receptor (Figure 9C). An increase in the potency of Pro^{1}TRH for the N289D receptor was also observed for stimulation of the $[Ca^{2+}]_i$ response in mammalian cells, demonstrating that the results obtained in *Xenopus* oocytes were not unique to the heterologous test system. Thus, complementary changes in the pyroglutamyl ring nitrogen of TRH and residue 289 of the receptor offer strong evidence to support a direct contact between the pyroglutamyl ring NH of TRH and Asn289 of the receptor. Because the pyroglutamyl ring NH of TRH is most likely a hydrogen bond donor, we propose a hydrogen bond between the pyroglutamyl ring NH of TRH and the carbonyl oxygen of Asn289 in the receptor, the ligand being the donor and the receptor being the acceptor (Figure 9A).

An important, but unlikely, consideration is that the binding site in the receptor for Pro^{1}TRH might be different from that of TRH and, therefore, identification of the contact site for Pro^{1}TRH would not give useful information on the binding site for TRH. Although this possibility cannot be

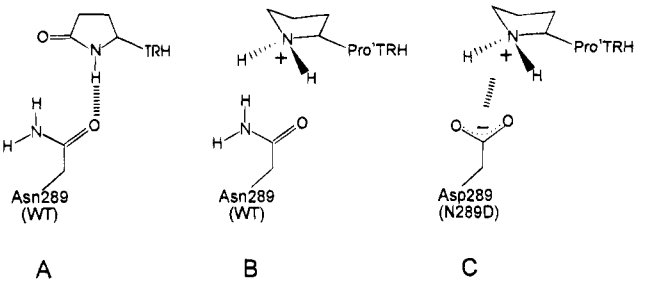

FIGURE 9: Schematic of TRH and Pro^{1}TRH interactions with Asn289 or Asp289 in the TRH receptor. (A) Hydrogen bonding between the pyroglutamyl ring NH in TRH and the carbonyl oxygen of Asn289 in the WT receptor is shown. There may be additional interactions between the pyroglutamyl ring of TRH and the receptor (not shown, see Discussion). (B) In Pro^{1}TRH, several of the hydrogen bond interactions proposed for the pyroglutamyl ring of TRH will be lost due to the absence of the carbonyl oxygen, change in the ring pucker, and the change in the electron orbital structure of the ring nitrogen. In addition, the presence of positive charge on the ring nitrogen contributes to destabilization of the Pro^{1}TRH–TRH receptor complex. (C) Introduction of a negative charge in the binding site for the pyroglutamyl ring of TRH creates an attractive force for the positively charged Pro^{1}TRH, thus explaining the increased potency of Pro^{1}TRH for the N289D receptor.

formally eliminated until the crystal structures are available for TRH–receptor and Pro^{1}TRH–receptor complexes, this possibility is highly unlikely. Pro^{1}TRH gives the same full agonist response at sufficiently high concentration as does TRH (Perlman et al., 1994b), and the dose–response curve for Pro^{1}TRH is parallel to that of TRH for both the WT and N289D receptors (Perlman et al., 1994b; and Figure 4). Furthermore, the relative difference in receptor binding affinities between TRH and Pro^{1}TRH (43 000-fold difference for the WT receptor) (Perlman et al., 1994b) correlates well with the relative difference in receptor activation between the two agonists (>10 000-fold but <100 000-fold for the WT receptor). These findings would be unlikely to occur if the binding site for Pro^{1}TRH was different from that for TRH. In addition, the lack of a further decrease in the potency of Pro^{1}TRH for the N289A receptor, in comparison to WT receptor, indicated that the interaction lost by modification of Asn289 was redundant with the interactions lost by modification of the pGlu ring of TRH, adding additional support for direct interaction between Asn289 and the pGlu of TRH. Therefore, we conclude that residue 289, which was identified as a binding site for Pro^{1}TRH (Figure 9C), is also a binding site for TRH and that the pGlu of TRH interacts with Asn289 in the WT receptor (Figure 9A).

Although the complementary modification approach between TRH and the receptor identified Asn289 in EL3 as a contact site for the pGlu of TRH, this result does not exclude additional contact sites for the pGlu in the receptor. Indeed, the experimental data suggest that the pGlu of TRH does make multiple concerted contacts with the receptor and that Asn289 is only one of these contact sites. Accordingly, modification of the pGlu of TRH would result in simultaneous loss of multiple interactions in the ligand–receptor complex while modification of the receptor at any one of the multiple contact sites would result in loss of only a single interaction. The much larger (>10 000-fold) decrease in the potency of Pro^{1}TRH for the WT receptor than the decrease in the potency of TRH for the N289D or the N289A receptors (100- or 10-fold, respectively) supports this conclusion.

Concerted interactions of the pGlu residue in TRH with multiple sites in the receptor also explain why the potency of Pro^{1}TRH for the N289D receptor was not as high as the potency of TRH for the WT receptor, and why TRH was a more potent agonist than the Pro^{1}TRH for the N289D receptor. Conversion of Asn289 to Asp interferes with the interaction of pGlu with Asn289, but it may not affect other interactions between pGlu and the receptor. Therefore, TRH maintains several of its multiple pGlu interactions with the receptor, while Pro^{1}TRH may have lost all of these interactions because of the multiple structural changes discussed in the Results (also see Figure 9B). Conversion of Asn289 to Asp could partially restore the lost interaction with Pro^1-TRH, but the multiple interactions between the pGlu of TRH and the WT receptor are missing in the interactions between Pro^{1}TRH and the N289D receptor.

Our results, which demonstrate a direct interaction of Asn289 in the TRH receptor with the pyroglutamyl ring of TRH, are not consistent with the model previously proposed by Perlman et al. (1994a,b). They concluded that Tyr106 and Asn110 in the putative third transmembrane helix interact directly with the carbonyl oxygen and the NH of the pyroglutamyl ring of TRH, respectively. On the basis of their expermental data and computer analysis, they proposed a model for the entire ligand binding pocket in which the orientation of TRH in the receptor is essentially the opposite of that which we propose (Perlman et al., 1994a). One might argue that the discrepancy between our results and those of Perlman et al. (1994a,b) is due to differences in the receptor environment between their experiments and ours. We used *Xenopus* oocytes while Perlman et al. used COS-1 cells to express WT and mutant TRH receptors. This explanation is unlikely to be correct because the relative potency of TRH to Pro^{1}TRH measured in *Xenopus* oocytes agrees well with that measured in the COS-1 cells (Perlman et al., 1994b). More important, we confirmed the increase in the potency of Pro^{1}TRH for the N289D receptor in GH_12C_1b mammalian pituitary cells by measuring $[Ca^{2+}]_i$ after Pro^{1}TRH treatment. We believe, as discussed below, that the discrepancy arises from a difference in the interpretation of the experimental data. Perlman et al. (1994a) converted Asn110 to alanine (N110A) or serine (N110S) and measured the affinity of MeTRH and Hgl^{1}TRH for the WT and mutant receptors. Hgl^{1}TRH differs from TRH by conversion of the NH in the pyroglutamyl ring to oxygen. Hgl^{1}TRH had 400-fold lower affinity for the WT receptor than TRH (Perlman et al., 1994a,b). They observed that the affinity of Hgl^{1}TRH was decreased only 6- or 10-fold for each of these mutants while the affinity of MeTRH was decreased 32- or 86-fold, respectively, suggesting incomplete additivity in the loss of binding energy with modification of the ligand and modification of the receptor. Perlman et al. (1994a) argued that the incomplete additivity was evidence for a direct interaction of Asn110 with the pyroglutamyl ring NH.

However, incomplete additivity is not strong evidence because a direct interaction of the pyroglutamyl ring NH with Asn110 would predict no additivity rather than incomplete additivity in the loss of binding energy and, therefore, no decrease in the affinity of Hgl^{1}TRH for the mutant receptors (N110A and N110S). In fact, the experimental data presented are fully consistent with an indirect effect of the Asn110 mutation on the overall structure of the binding pocket. To test the model of Perlman et al. (1994a) for a direct interaction of Asn110 in the putative third transmembrane helix with the pGlu ring NH of TRH, we converted Asn110 to aspartic acid (N110D mutant) and measured activation of the mutant receptor with TRH and Pro^{1}TRH. Based on the same logic as applied to the N289D receptor, the potency of Pro^{1}TRH for the N110D receptor would be expected to increase due to the charge interaction, if Asn110 made a direct contact with the pyroglutamyl ring NH of TRH. One might raise a question about the feasibility of such an experiment, considering the transmembrane location of the Asn110 as compared to the presumed extracellular location of Asn289. For example, Asp110 may remain protonated and Pro^{1}TRH may become deprotonated in the hydrophobic environment of the receptor. Although such a possibility cannot be ruled out, it is unlikely because another transmembrane acidic residue in the TRH receptor, Asp71, appears to be deprotonated. Conversion of this residue to Asn severely impaired signal transduction (B. Han and A. H. Tashjian, Jr., unpublished data). In addition, precedents for a strong charge interaction in the hydrophobic transmembrane environment can be found in other G protein-coupled receptors, such as the catecholamine receptor (reviewed in Strader et al., 1994) and bovine rhodopsin (Zhukovsky & Oprian, 1989). Attractive charge interactions, within the hydrophobic transmembrane environment, between the negatively charged Asp113 of the β-AR and the protonated amine of the ligand, provide a major binding energy, disruption of which by conversion of Asp113 to Ser or Ala resulted in a 10 000-fold decrease in agonist and antagonist affinity (Strader et al., 1991). Thus, considering the hydrophobic environment of Asn110 in the TRH receptor, a potential charge interaction between Asp110 in the N110D receptor with Pro^{1}TRH would be expected to be quite strong, and a large increase in the potency of Pro^{1}TRH would be expected for the N110D receptor. However, the potency of Pro^{1}TRH for the N110D receptor was actually decreased >10-fold while the potency of TRH did not change significantly (<10-fold). Both the absolute potency of Pro^1-TRH and the relative potency of Pro^{1}TRH to TRH were decreased for the N110D receptor. These results were in clear contrast to the results obtained with the N289D receptor, which showed an increase in both the absolute and relative potency of Pro^{1}TRH. Because maximal responses to TRH from oocytes expressing the N110D receptor were fully comparable to those in oocytes expressing the WT receptor, and because the potency of TRH was not significantly decreased, receptor expression or the global structure of the receptor does not appear to be significantly changed. More importantly, if Asn110 made a direct contact with the pyroglutamyl ring of TRH, one would expect an increase in the relative potency of Pro^{1}TRH to TRH for the N110D receptor even if the absolute potency of Pro^{1}TRH were decreased due to such indirect effects. This was not observed. Therefore, we found no experimental evidence to support a direct interaction of Asn110 with the pyroglutamyl ring NH of TRH.

To show an interaction of Tyr106 with the pyroglutamyl carbonyl oxygen of TRH, Perlman et al. (1994a) replaced Tyr106 with Phe (Y106F mutant) and measured activation of the WT and Y106F receptors by TRH and Pro^{1}TRH. From the EC_{50} values for stimulation of inositol polyphosphate formation, they estimated the affinities of TRH and Pro^1-TRH for the WT and Y106F receptors. They observed that,

compared to the affinity of TRH for the WT receptor, the affinity of TRH for the Y106F receptor and the affinity of Pro[1]TRH for the WT receptor were decreased 1.0×10^5-fold and 1.1×10^5-fold, respectively, and that the affinity of Pro[1]TRH for the Y106F receptor was decreased 16×10^5-fold. They argued that the incomplete additivity of the loss of binding energy (or multiplicity in the fold decrease in affinity) strongly supported a direct interaction between the pyroglutamyl carbonyl oxygen of TRH and the hydroxyl group of Tyr106 in the receptor. They attributed a contribution of 1×10^5-fold difference in binding affinity (equivalent to 7 kcal/mol in binding energy) to this single hydrogen bond.

In their analysis of the experimental data, the multiple structural changes in Pro[1]TRH (Figure 1) including the effect of the positive charge were not considered. This reasoning led to an unrealistically high estimate for the effect of removal of the carbonyl oxygen from the pGlu ring of TRH. Results on the thioamide analog of the pGlu ring of TRH, in which the carbonyl oxygen was converted to sulfur, indicate that the binding energy lost by the loss of a hydrogen bond with the carbonyl oxygen of the pGlu ring of TRH is small (Lankiewicz et al., 1992). Given the large difference in the enthalpy of vaporization between H_2O and H_2S (>5 kcal/mol), the strength of a hydrogen bond to sulfur is much weaker than the equivalent hydrogen bond to oxygen (Mahan, 1979). Because the thioamide analog is isosteric with TRH, and yet the hydrogen bond to the carbonyl oxygen of the pGlu ring of TRH is eliminated, comparison of the binding affinity of the thioamide analog to that of TRH would give a more accurate estimation of the contribution of this hydrogen bond. In fact, the thioamide analog of TRH showed no decrease in its potency for stimulation of TSH secretion from the rat pituitary gland, and only a 4-fold decrease in receptor binding affinity as compared to TRH (Lankiewicz et al., 1992). Therefore, most of the 1.1×10^5-fold decrease in the affinity of Pro[1]TRH for the WT receptor can be explained by the multiple structural changes in the Pro[1]TRH molecule. We believe that the interpretation of their experimental data by Perlman et al., which did not consider the multiple structural differences between TRH and Pro[1]TRH, is not convincing. We, therefore, propose that the role of Tyr106 in TRH binding is indirect.

In summary, we present new evidence for a direct interaction of Asn289 in the putative third extracellular loop of the TRH receptor with its agonist tripeptide TRH and propose a hydrogen bonding interaction of Asn289 with the lactam moiety of the pyroglutamyl ring of TRH (Figure 9A). We conclude that TRH binds, at least in part, to the extracellular domain of its receptor rather than entirely within the transmembrane helices. Transmembrane residues that have been previously identified to affect TRH binding may do so by indirect effects on overall receptor structure; however, studies on these residues may eventually prove useful in understanding the mechanism of signal transduction initiated by ligand binding. The TRH receptor serves as a particularly useful model system to study signaling mediated by a small peptide ligand which binds to an extracellular domain of its G protein-coupled receptor.

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