Altered Ligand Dissociation Rates in Thyrotropin-Releasing Hormone Receptors Mutated in Glutamine 105 of Transmembrane Helix III[†]

Donato del Camino, Francisco Barros, Luis A. Pardo, and Pilar de la Peña*

Departamento de Bioquímica y Biología Molecular, Facultad de Medicina, Universidad de Oviedo, 33006 Oviedo, Spain

Received September 6, 1996; Revised Manuscript Received November 12, 1996[⊗]

ABSTRACT: Glutamine 105 in the third transmembrane helix of the thyrotropin-releasing hormone receptor (TRH-R) occupies a position equivalent to a conserved negatively charged residue in receptors for biogenic amines where it acts as counterion interacting with the cationic amine moiety of the ligand. Maximum levels of response to TRH in oocytes expressing wild-type TRH-Rs were indistinguishable from those of oocytes expressing receptors mutated to Glu, Asn, or Asp in position 105. However, the EC_{50} values for activation of oocyte responses increased more than 500 times in oocytes expressing mutant Glu^{105} receptors, in which the amido group of Gln^{105} has been removed by site-directed mutagenesis. Charge effects do not seem to be involved in the huge effect of mutating Gln^{105} to Glu, since mutation of Gln^{105} to Asp induces only a 15-fold increase in EC_{50}. Furthermore, no change in EC_{50} is observed after mutation of Asn^{110} to Asp. The affinity shift (identified by changes in EC_{50} values for systems of comparable efficacy) in Glu^{105} mutant receptors was partially recovered in oocytes expressing Asn^{105} mutant receptors. These results and those obtained after substitution of Lys, Leu, Tyr, and Ser for Gln^{105} suggest that the presence and the correct position of the Gln hydrogen bond−donor amido group are important for normal functionality of the receptor. In wild type or Asp^{105} mutant receptors showing the same maximal responses, decreases in affinity with TRH and methyl-histidyl-TRH correlated with increased dissociation rates of hormone from the receptor. Rapid dilution experiments following subsecond stimulation indicate that the TRH-R is converted rapidly from a form showing fast dissociation kinetics to a form from which the hormone dissociates slowly. Mutation of residue 105 impairs the receptor shift between these two forms. This effect was demonstrated in a direct way by comparing [^{3}H]methyl-histidyl-TRH dissociation rates in COS-7 cells transfected with either wild type or Asp^{105} mutant TRH-Rs. Thus, residues located in transmembrane helix III positions equivalent to those of the counterions for biogenic amines, regulate hormone−receptor interactions in the TRH receptor (and perhaps other receptors). Furthermore, the nature of the amino acid in these positions may also play a role, directly or indirectly, in conformational changes leading to receptor activation, and hence to signal transduction.

Cloning of the thyrotropin-releasing hormone receptor (TRH-R)[1] indicates that it is a member of the seven-transmembrane-spanning, G protein-coupled receptor family (Straub et al., 1990; de la Peña et al., 1992; Zhao et al., 1992). Several features about ligand binding and activation of receptors in this family have been derived from studies on rhodopsin and receptors for biogenic amines such as adrenaline, acetylcholine, serotonin, histamine, and dopamine. In these cases, extensive site-directed mutagenesis, analysis of affinity-labeled receptor proteins, and parallel engineering of chemical groups in the receptor and ligand molecules, identified an acidic residue in transmembrane helix III (TM-III) as critical for the initial ion−ion interactions between the positively charged amino head group of the amines and the receptor [for reviews see Strosberg (1991), Dohlman et al. (1991), Ostrowski et al. (1992), Savarese and Fraser (1992), and Wess (1993)]. This assignment was further supported by early structural studies showing that such a negatively charged residue was present in all known G protein-coupled receptors that bind protonated amine ligands, but not in those receptors whose ligands are not protonated amines (Strosberg, 1991; Strader et al., 1988, 1989, 1991). It has been proposed that the acidic residue in TM-III participates in a charge relay mechanism leading to receptor activation (Chung et al., 1988; Saunders & Freeman, 1989). This hypothesis was subsequently challenged by studies on β2-adrenergic receptors mutated in Asp^{113} to Ser. This mutant receptor was fully active providing that the amine function of the ligand is also substituted by chemical groups able to form hydrogen bonds with the mutated Ser^{113} (Strader et al., 1991). It has been argued also that the role of the Asp residue in TM-III of amine ligand receptors is not to dock the cationic head group of agonists, but to participate in receptor activation and subsequent formation of a receptor ternary complex agonist−receptor−G protein (Hulme et al., 1993). In the TRH-R, a Gln is located in position 105, equivalent to that occupied by the counterion Asp in TM-III of biogenic amine receptors. Recent elucidation of the primary structure of a number of G protein-coupled receptors indicates that most of the receptors for ligands that are not small protonated amines lack a negatively

[†] This investigation was supported by Grants PB90-0789 and PB93-1076 from DGICYT of Spain, an Incorporation Contract from Spanish MEC to LAP and a fellowship from FICYT of Asturias (Spain) to D. del C.

* Corresponding author. Phone: 34-8-5103565; Fax: 34-8-5103534. E-mail: lapf@bioaxp.quimica.uniovi.es.

[⊗] Abstract published in *Advance ACS Abstracts,* February 15, 1997.

[1] Abbreviations: TRH, thyrotropin-releasing hormone (pGlu-His-ProNH$_2$); TRH-R, TRH receptor; Met-TRH, methyl-TRH (pGlu-3-methyl-His-ProNH$_2$); WT, wild-type; PBS, phosphate-buffered saline solution; TM-III, transmembrane helix III; CDPX, chlordiazepoxide.

S0006-2960(96)02253-2 CCC: $14.00

charged residue in an equivalent position of TM-III. The few exceptions to this rule include receptors for interleukin-8, somatostatin, chemotactic peptide, opiate drugs, and calcitonin (Thomas et al., 1990; Lin et al., 1991; Yamada et al., 1992; Evans et al., 1992; Leong et al., 1994). Substituting Glu by Ala in TM-III of interleukin-8 receptors does not modify ligand binding nor signal transduction (Leong et al., 1994). Nevertheless, few mutagenesis studies have been performed in receptors that, like the TRH-R, lack a negatively charged acidic residue in an equivalent position of TM-III. Early work on the mouse TRH receptor by exhaustive mutagenesis of residues located along TM-III, including Gln^{105}, allowed the elaboration of a model for the receptor binding pocket and for participation of different amino acids in ligand−receptor interaction (Perlman et al., 1994a, 1995). According to it, TRH would bind entirely within the transmembrane helices. A direct interaction of Tyr^{106} and Asn^{110} in TM-III and TRH constitutes an essential feature of this model. More recently, a model in which TRH binds to its receptor in the opposite orientation to that previously proposed has been elaborated also (Han & Tashjian, 1995a,b). In this case, binding of TRH is located mainly to the extracellular domain of the receptor. Modification of key residues on TM-III would affect ligand−receptor interaction by indirect effect on overall receptor structure. An important implication of this model would be that careful characterization of functional properties of receptors modified in key TM-III residues can be useful not only to know the implication of certain amino acids in the structure of the binding site, but also to understand the mechanism of signal transduction initiated by ligand binding. In this report, the effect of different substitutions of Gln^{105} on the TRH receptor is studied. Our results demonstrate that the presence of Gln at position 105 is important for normal functionality of the receptor and that mutations in this residue alter rates of ligand dissociation. This indicates that, besides its important role as counterion in biogenic amine receptors, the amino acid located at this position in other receptors can regulate hormone−receptor interactions. The possibility that the nature of the residue in this position cal also play a role, directly or indirectly, in receptor conformational changes involved in signal transduction is discussed.

MATERIALS AND METHODS

Materials. Cell culture plasticware was obtained from Nunc. Tissue culture media were purchased from Sigma Chemical Co. Sera and other culture reagents were from Seromed. TRH, Met-TRH, CDPX, and chloroquine were obtained from Sigma. [^{3}H]Met-TRH (50−90 Ci/mmol) was purchased from Dupont NEN. All other chemicals were of the highest purity available and purchased from Sigma, Pharmacia, and Boehringer Mannheim.

Mutagenesis and Preparation of cRNA. Isolation and handling of the TRH-R cDNA have been described previously (de la Peña et al., 1992a). Site-directed mutagenesis was performed on the full-length, GH_3 cell TRH-R cDNA inserted in pBluescript II SK^- (Stratagene). Single stranded DNA generated with the helper phage R408 was used for oligonucleotide-directed mutagenesis. Annealing of phosphorylated mutagenic oligonucleotide to single-stranded DNA template, primer extension, ligation and transformation of XL-1 Blue *Escherichia coli* cells were performed according to the recommended Stratagene protocols. The screening of mutants was done by colony hybridization using the radiolabeled mutagenic oligonucleotide as a probe. The identity of the desired mutations was verified by dideoxy sequencing of the mutant plasmids.

Microinjection and Electrophysiology of Oocytes. Procedures for microinjection and electrophysiology of oocytes have been described elsewhere (de la Peña et al., 1992a,b). Oocytes were maintained and studied in OR-2 medium (in mM: NaCl, 82.5; KCl, 2; $CaCl_2$, 2; $MgCl_2$, 1; Na_2HPO_4, 1; HEPES, 10; titrated to pH 7.5 with NaOH). Cytoplasmic microinjections were performed with 20−50 nL of *in vitro* synthesized cRNA per oocyte. Responses were studied in oocytes manually deprived of the follicular membranes as described (de la Peña et al., 1992b). Functional expression was assessed 1−2 days after microinjection. Whole-cell current measurements were performed using the two-electrode voltage-clamp method with a Turbo TEC 01C (NPI, Tamm, Germany) amplifier and 2 M KCl-filled glass capillaries showing DC resistances between 0.5 and 3.0 MΩ. Membrane potential was clamped at −60 mV. The resting membrane potential of the oocytes measured after insertion of the potential electrode ranged between −30 and −60 mV. Oocytes showing either membrane potentials below −20 mV after impalement with the second electrode or holding currents bigger than 200 nA once voltage clamped were discarded. The magnitude of the response was obtained measuring the amount of current in the initial maximum following the introduction of the agonist in the recording chamber. Such a maximum was sometimes flanked by smaller oscillations delineating additional peaks and valleys superposed to the main response. In those cases, current magnitude was measured at the highest value of inward current, which systematically appeared within 10 s following entrance of the agonist to the chamber. Sometimes, mainly at low subsaturating ligand concentrations and with small magnitude second responses, delayed and slow oscillating responses were elicited. In these cases the maximum value of inward current was considered, irrespective of its position along the time course. Current recordings were routinely obtained in an experimental chamber of 0.2 mL volume continuously perfused at 4.5−5.0 mL/min. For experiments in which short expositions to hormone were performed, a home-made chamber was used to apply directly the ligand to the oocyte surface. The flow through the chamber was maintained at a rate of 3−5 mL/min all along the experiment using a peristaltic pump. The introduction of hormone-free saline was stopped by switching the output of the pump to a hormone-containing tubing with its end positioned within 1 mm from the oocyte surface. For this purpose, a small three-way electronic valve connected to a timer was interposed between the pump and the chamber. Subsequent switch of the pump output back to the hormone-free tubing ensures the rapid elimination of the ligand from the oocyte surrounding area. Such a rapid elimination of the ligand from the oocyte area is also ensured by building the chamber as a long narrow channel to obtain a laminar flow through it. Thus, the dimensions of the channel limited by the chamber walls were calibrated to leave only a small space surrounding the oocyte surface. In this way, switching the output of the pump back to the hormone-free tubing attached to the end of the chamber ensures the almost instantaneous and complete removal of the ligand from the oocyte surface. By limiting the switch of the electronic valve as desired, it is possible to limit the application of the ligand even to the subsecond time span.

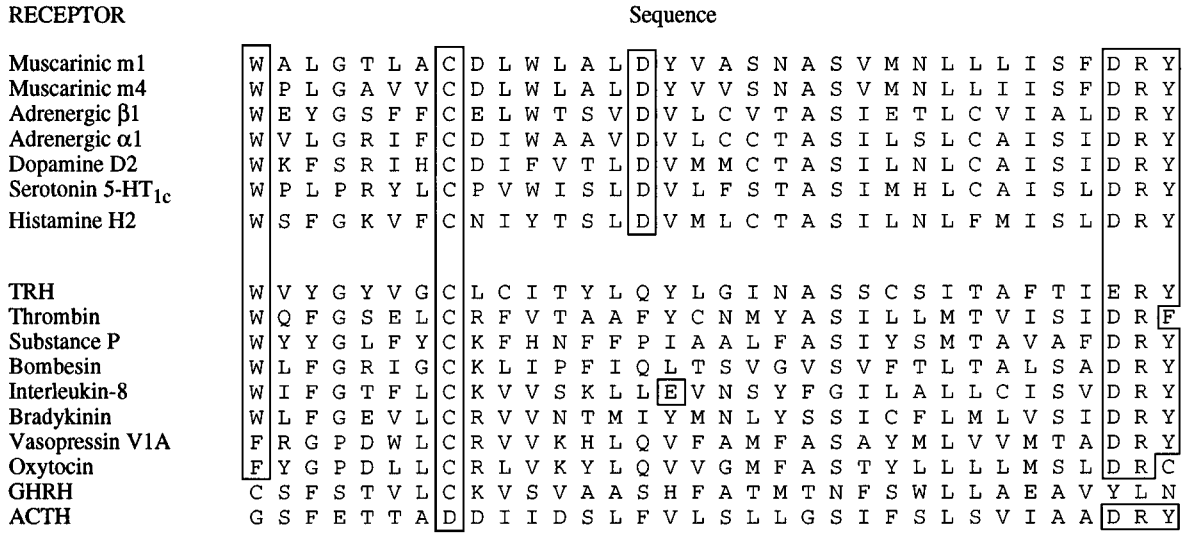

FIGURE 1: Amino acid sequence of the putative third transmembrane domain region of G protein-coupled receptors for biogenic amines (top) and receptors for other ligands (bottom). The position of a highly conserved Trp residue among G protein-coupled receptors, a highly conserved Cys at the carboxyl end of extracellular loop II and the Glu-Arg-Tyr canonical sequence at the amino end of intracellular loop II are boxed. Also boxed is shown the Asp residue characteristic of the biogenic amine receptors, which is replaced with an uncharged amino acid in most other receptors. One letter amino acid code is used. GHRH means growth-hormone releasing hormone. ACTH stays for adrenocorticotropic hormone. Receptor sequences correspond to those referenced as follows: muscarinic m1 and m4 (Bonner et al., 1987); adrenergic β1 (Machida et al., 1990); adrenergic α1 (Cotecchia et al., 1988); dopamine D2 (Bunzow et al., 1988); serotonin 5-HT-1c (Julius et al., 1988); histamine H2 (Gantz et al., 1991); TRH (de la Peña et al., 1992a); thrombin (Vu et al., (1991); substance P (Yokota et al., 1989); bombesin (Battey et al., 1991); interleukin-8 (Leong et al., 1994); bradykinin (Yamada et al., 1992); vasopressin V1A (Morel et al., 1992); oxytocin (Kimura et al., 1992); GHRH (Gaylinn et al., 1993); ACTH (Mounjoy et al., 1992).

Cell Culture and Transfection. WT and mutant TRH receptors were transiently expressed in COS-7 cells obtained from the American Type Culture Collection. Cells were grown in Dulbecco's modified Eagle's medium nutrient mixture Ham's F-12 (1:1 mixture, Sigma) containing phenol red and supplemented with 0.13% $NaHCO_3$, 50 units/mL penicillin, 50 μg/mL streptomycin, and 10% fetal bovine serum. For transfection experiments cells were trypsinized and grown 12−16 h to semiconfluence in 60 mm dishes. Cells were deprived of growth medium immediately before transfection by washing them twice with phosphate-buffered saline (PBS). Transfection mixture contained 0.4−2.0 μg of DNA and 300 μg /mL of DEAE-dextran in PBS. This mixture (240 μL per dish) was applied dropwise to each dish and gently swirled. After 30 min at 37 °C, 2.5 mL of growth medium without serum containing 0.16 mM chloroquine was added to the dishes. The chloroquine-containing medium was removed by aspiration after 3 h at 37 °C. Cells were subsequently incubated at 37 °C in growth medium until use.

Binding of [^{3}H]Methyl-TRH to Transfected Cells. Binding assays were performed 48−72 h after transfection in triplicate samples using the 60 mm dishes used for transfection. Cells were washed twice with cold PBS and then incubated with 600 μL per dish of growth medium containing [^{3}H]Met-TRH. 1 and 4 nM [^{3}H]Met-TRH were used for binding to cells transfected with WT or Gln^{105}-mutated receptors, respectively. Nonspecific binding was measured in the presence of 10 μM unlabeled TRH. The amount of [^{3}H]Met-TRH bound was determined at the indicated times washing the dishes three times with cold PBS and scraping the cells into 1 mL of buffer containing 150 mM NaCl, 1% Nonidet P40, 0.1% sodium dodecyl sulfate, 0.5% deoxycholate, and 50 mM TRIS-HCl titrated to pH = 8.0. The radioactivity that remained associated to cells was then quantified by scintillation counting. Under the binding conditions used, total binding ranged among 1000−5000 cpm, and nonspecific binding was 100−200 cpm.

Statistical Analysis of the Data. Due to significant differences in response levels found in populations of oocytes from different frogs, a multifactor analysis of variance was applied to the data (de la Peña et al., 1995). This allowed us to make the effect of the mutations independent from those caused by the differences found in oocytes from different animals. Furthermore, only data of oocytes from the same donors expressing either WT or mutant receptors were used to compare the effect of an amino acid change on a given response parameter. Careful comparison of >200 oocytes injected with WT receptor cRNA indicated that a logarithmic transformation was necessary to obtain a normal distribution of response magnitudes at the initial maximum. Subsequently, data normalized by this procedure were used for determination of significance levels.

RESULTS

Effect of Gln^{105} Mutations on Responses Induced by TRH in Oocytes Expressing TRH Receptors. Figure 1 shows the amino acid sequences of the putative third transmembrane domain for a number of G protein-coupled receptors cloned to date. It can be seen that every receptor for all five different biogenic amine ligands contains an aspartate residue in the same position of that domain. However, most of the receptors that do not bind a protonated amine lack a charged residue in an equivalent position. These include the TRH-R carrying a Gln residue in position 105. As an initial approach to investigate the role of the Gln^{105} side chain on functionality of the TRH-R a Glu residue was introduced at this position. This replaced the amide side chain of Gln with its corresponding acid. The functional properties of the mutant receptor were subsequently studied after expression in *Xenopus* oocytes. As shown in Figure 2 and Table 1, the magnitude of the responses to TRH was similar in oocytes expressing either WT or Glu^{105} mutant receptors. However, a much higher hormone concentration was necessary to elicit maximal responses in oocytes injected with Glu^{105} mutant

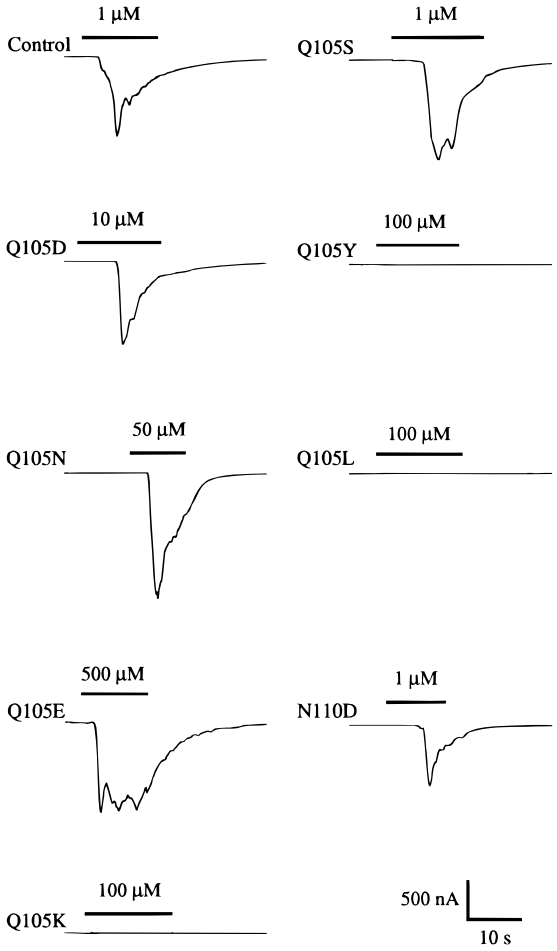

FIGURE 2: Functional responses to TRH of oocytes expressing wild-type (Control) or several mutant TRH receptors. Representative inward Cl^- currents evoked in voltage-clamped oocytes 2−3 days after microinjection with receptor cRNA are shown. The duration of perfusion with saline plus the indicated TRH concentrations is indicated by horizontal lines above the traces. The type of receptor being expressed is indicated on the left, with the first and last letters meaning the residue (in one-letter code) present in the WT receptor and the amino acid to which it has been changed in the mutant receptor, respectively. The number between both letters identifies the position of this residue starting at the amino terminus of the GH_3 cell TRH-R (de la Peña et al., 1992a).

receptor cRNA (Figure 3 and Table 2). Interestingly, no responses were observed upon addition of serotonin, dopamine, acetylcholine, adrenaline, or noradrenaline up to 100 μM (not shown). This indicates that, besides the ion−ion interaction between the positively charged amino group of these ligands and the receptor acidic residue in helix III, additional interactions dictate the binding and/or activation of different receptors by their ligands. These interactions are not present between the TRH-R and the tested biogenic amines.

Conversion of Gln^{105} to Glu introduces a slightly smaller side chain and retains a hydrogen bond acceptor. However it also changes charge and converts a bifunctional hydrogen bond acceptor and donor in only an acceptor. To test the possible implications of these changes individually, residue 105 was changed to different amino acids. These include Asn, Asp, Lys, Leu, Tyr, and Ser. Conversion of Gln to Asn maintains the functional properties of the Gln side chain and only alters its size. The Gln^{105} to Asp mutant would help to clarify the effect of introducing a negative charge, but a shorter side chain is also introduced and the bifunctional amide is replaced by an exclusively hydrogen bond acceptor.

Table 1: Magnitude of Responses Induced by TRH in Oocytes Expressing Wild-Type (WT) or Different Mutant TRH Receptors[a]

receptor	response (nA)
WT	1240 ($n = 18$) (771−1709)
Glu^{105}	2231 ($n = 15$) (1718−2745)
WT	2442 ($n = 22$) (1594−3290)
Asn^{105}	3851 ($n = 22$) (3003−4699)
WT	1105 ($n = 70$) (915−1294)
Asp^{105}	1247 ($n = 50$) (1023−1471)
WT	2468 ($n = 9$) (1751−3186)
Ser^{105}	2336 ($n = 11$) (1687−2985)
WT	1273 ($n = 7$) (726−1820)
Asp^{110}	955 ($n = 11$) (519−1391)

[a] Averaged responses in the initial maximum are presented with 95% confidence intervals shown in parentheses below the response levels. Total number of oocytes analyzed in each case is indicated by *n*. As stated in Methods, a multifactor analysis of variance was applied to the data in order to make the effects of the mutations independent from those caused by the significant differences found in populations of oocytes from different frogs. Furthermore, only data of oocytes from the same donors are used to compare the responses in WT or mutant receptors. No significant differences were found in the levels of response elicited with any mutant receptor *vs* those obtained with WT receptors. TRH was used at 1 mM except for oocytes expressing Asp^{105}, Asn^{105}, and Glu^{105} mutant receptors, challenged with 10, 50, and 500 μM, respectively.

A positive charge is introduced in the Gln^{105} to Lys mutant. Deletion of all hydrogen bonding active groups is achieved by substitution of Leu for Gln^{105}. The hydrogen bond donor is maintained after Gln^{105} to Tyr or Ser change, but its spatial localization is altered. As an additional control of the effect caused by introduction of a negatively charged residue along the hydrophobic transmembrane domain III, Asn^{110} was also changed to Asp. Finally, since Tyr^{106} has been recently implicated in a direct interaction between TRH and its receptor (Perlman et al., 1994b), we studied also the effect of converting it to Phe either alone or in combination with changes in Gln^{105}. Some of the functional consequences of these changes are described below.

The results obtained with the Gln^{105} to Glu mutant (see above) could indicate that the presence of a negative charge in the third transmembrane domain is deleterious for the normal functionality of the receptor. Functional expression of receptors in which Gln^{105} is changed by Asp indicates that this is not the case. As it is shown in Table 1, maximal responses to TRH in oocytes expressing Asp^{105} mutant receptors were the same as those obtained with WT receptors. Furthermore, the EC_{50} values for activation of responses to TRH were only about 15-fold higher than those obtained in oocytes expressing WT receptors (Table 2). This contrasts with the more than 500 times higher EC_{50} value obtained after changing Gln^{105} to Glu and indicates that introduction of a negatively charged residue in the third transmembrane domain is not causing the huge increase in EC_{50} values observed with the Glu^{105} mutant. Further support to this interpretation came from results obtained with the Asn^{110} to

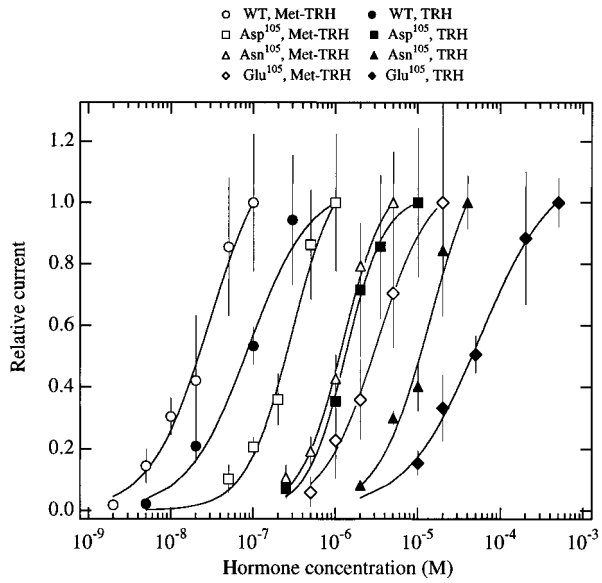

FIGURE 3: Concentration dependence of TRH- and Met-TRH-induced responses. Stimulation of inward Cl^- currents by TRH (filled symbols) and Met-TRH (open symbols) in oocytes expressing wild-type (WT, circles) or Glu^{105} (diamonds), Asn^{105} (triangles), and Asp^{105} (squares) mutant receptors is represented in the graph. Current magnitudes in the initial maximum normalized to maximal current at the higher ligand concentration used are represented on the ordinates. Values are presented as mean ± SEM for two to four experiments. Curves were fitted and draw to equations of the type

$$y = I_{max}\{1 - [1/(1 + (x/EC_{50}^{h}))]\}$$

where I_{max} is the maximum current value at saturating hormone concentrations, EC_{50} stands for the concentration required to achieve half-maximal response, and h is the Hill coefficient.

Table 2: Activation Parameters of Wild-Type (WT) and Residue 105 Mutant TRH Receptors by TRH and Met-TRH[a]

receptor	agonist	EC_{50} (nM)
WT	Met-TRH	31 ± 12 ($n = 3$)
	TRH	87 ± 8 ($n = 4$)
Glu^{105}	Met-TRH	2 450 ± 750 ($n = 2$)[b,c]
	TRH	49 386 ± 1 280 ($n = 3$)[b,c]
Asn^{105}	Met-TRH	1235 ± 2 ($n = 2$)[b]
	TRH	14 767 ± 659 ($n = 2$)[b]
Asp^{105}	Met-TRH	427 ± 140 ($n = 3$)[b]
	TRH	1 309 ± 170 ($n = 3$)[b]
Ser^{105}	TRH	34 ± 166 ($n = 2$)

[a] The EC50 values for oocyte responses correspond to the data shown in Figure 3. Data are expressed as mean ± SEM for the number of experiments indicated in parentheses. [b] Significantly bigger than WT values ($p < 0.05$). [c] Significantly bigger than Asn^{105} values ($p < 0.05$).

Asp mutant in which maximal response levels and EC_{50} values were similar to those obtained with WT receptors (Table 1).

The situation of the hydrogen bond acceptor in Gln^{105} is not altered by substituting this amino acid residue with Glu. However, besides introducing a negative charge, mutation to Glu also removes a hydrogen bond−donor amido group. Substitution of Asn for Gln at position 105 yielded a receptor that showed a maximum level of oocyte responses similar to that of WT receptors (Figure 2 and Table 1). Furthermore, the EC_{50} value of activation by TRH in oocytes expressing Asn^{105} mutant receptors was significantly lower than that obtained with the Glu^{105} mutant (Table 2). This emphasizes the important role of the amido group in normal functionality of the receptor. However, since the EC_{50} values for TRH and Met-TRH are still 160- and 40-fold higher than those of oocytes expressing WT receptors (Table 2), it also indicates that the correct position of such a group is an important determinant of its role on the receptor. As it would be discussed later, this could also explain the apparently paradoxical results obtained with the Asp^{105}, Tyr^{105}, and Ser^{105} mutants.

Undetectable levels of response were achieved with the Lys^{105}, Tyr^{105}, and Leu^{105} mutant receptors (Figure 2). If this is due to lack of expression of these mutant receptors on the membrane or to the presence of inactive receptors remains to be established. Substitution of Gln^{105} by Ser did not alter the maximum level of oocyte responses. On the other hand, the EC_{50} value was also not significantly increased (from 87 nM in WT to 349 nM in Ser^{105} mutant receptors, see Table 2) by the mutation.

Conversion of Tyr^{106} to Phe yields a receptor able to activate oocyte responses only 40% of those obtained with WT receptors, but only at TRH concentrations as high as 10 mM (not shown). This response is abolished in a double mutant receptor carrying an additional Gln^{105} to Asp mutation. Such behavior of the Phe^{106} mutant receptor has been previously reported as indicative of a direct interaction between Tyr^{106} and the pyroglutamyl moiety of TRH [Perlman et al., 1994b; but see Han and Tashjian (1995a,b)]. Providing that lack of any detectable activity in the double mutant is not due to a complete absence of receptor expression, our results would indicate that an additive effect on receptor functionality is exerted by mutations in residues 105 and 106.

Alterations in Affinity of Ligands Induced by Mutation of the TRH Receptor at Gln^{105}. In the absence of spare receptors, a decrease in receptor number causes a decrease in the maximal response to agonists without a significant change in agonist potency. On the other hand, a decrease in receptor number in the presence of spare receptors causes a decrease in the potency of agonists without affecting the maximal response (Limbird, 1986). It has been shown that the number of expressed receptors determines the amplitude of oocyte responses to TRH (Straub et al., 1989; Matus-Leibovitch et al., 1994). Furthermore, maximal responses to TRH in different cell variants are proportional to cell surface receptor number (Ramsdell & Tashjian, 1985, 1986; Gershengorn et al., 1994), demonstrating a non-spare receptor model for TRH action. Thus, providing that the efficacies of the systems compared are the same, relative potency of activation (EC_{50}) would reflect relative affinities (Perlman et al., 1994b, 1995; Han & Tashjian, 1995a,b). Failure to reach a level of mutant receptors similar to that achieved with WT receptors would complicate interpretation of the data, and the possibility that a given receptor variant is overexpressed without variation in the maximal oocyte response cannot be excluded. Attempts to quantify the number of receptors using [^{3}H]Met-TRH binding to whole oocytes or oocyte membranes yielded very variable results and were not pursued further (not shown). On the other hand, mutant receptors with affinities for Met-TRH lower than WT by more than 1 or 2 orders of magnitude cannot be detected using [^{3}H]Met-TRH binding assays. Nevertheless, binding analysis of receptors expressed in transfected COS-7 cells indicates that the number of Asp^{105} mutant receptors is slightly bigger than that of WT receptors and that an affinity shift almost identical to that observed in EC_{50}

for oocyte responses is induced by the mutation (data not shown). It is also unlikely that the huge decreases in potency observed with the other residue 105 mutants are caused by a change in efficacy that does not affect maximal stimulation. Thus, a significant decrease in potency combined with a similar maximal response was taken as an initial evidence for a large decrease in affinity and not a lack of receptor expression. A comparison of the kinetic parameters of response to TRH and Met-TRH (a TRH analog with higher affinity than TRH for GH_3 cell native receptors; Hinkle, 1989) in oocytes expressing WT and mutant receptors is shown in Figure 3 (see also Table 2). Dose−response curves for TRH are displaced about 1, 2, and 2.5 orders of magnitude to higher values in the Asp^{105}, Asn^{105}, and Glu^{105} mutants, respectively, as compared with WT receptors. In each case, although maximum levels of response were similar, the EC_{50} values for Met-TRH were nearly one order of magnitude lower than those for TRH. Nevertheless, the relative potency of activation for the different receptors was maintained. This indicates that, regardless of the type of alterations caused by the mutations in the structure of the receptor, they similarly affect its interaction with both TRH and Met-TRH.

We also measured the potency of inhibition of TRH responses by the competitive TRH antagonist CDPX (Hinkle, 1989). Consistent with previous results (de la Peña et al., 1992a,b) the response of WT receptor-injected oocytes to 1 μM TRH decreased to 34% ± 6% ($n = 6$) and 22% ± 1% ($n = 11$; $N = 2$) in the presence of 20 and 100 μM CDPX (CDPX/TRH molar ratios of 20 and 100), respectively. In oocytes expressing Asp^{105} mutant receptors challenged with 5 μM TRH, similarly decreased levels of response [46% ± 6% ($n = 6$) and 18% ± 2% ($n = 10$; $N = 2$)] required CDPX concentrations of 100 and 500 μM, respectively. Thus, not only agonists but also competitor interactions with the receptor seem to be similarly impaired by substitution of residue 105.

Effect of Gln^{105} Mutations on Oocyte Response Recovery after Two Successive Additions of Hormone. As stated above, the increases in EC_{50} caused by mutations should arise from a parallel loss of ligand−receptor affinities. In a simple scheme this would also implicate that association and/or dissociation kinetic constants for interaction of ligand with its receptor should be decreased and increased, respectively, after replacement of residue 105. To gain further insights about the effect of mutations on association and dissociation parameters, two types of measurements were performed. First, the delay between addition of TRH and onset of responses was studied in oocytes expressing either WT or Asp^{105}, Asn^{105}, and Glu^{105} mutant receptors that showed similar levels of maximal response. Second, the magnitude of responses to two successive additions of hormone was measured.

The latency period between the application of TRH and the onset of the response was not significantly different in oocytes injected with either WT or residue 105 mutant receptor cRNAs. Furthermore, we never found any differences in the time necessary to reach the initial maximum of response (not shown). As discussed below, this suggests that big association rate increments do not seem to be the main reason for the huge decrements in affinity found with some of the mutated receptors.

Ligand−receptor dissociation parameters can be studied after binding of labeled ligand and subsequent washout. As stated above, we failed to detect reproducibly [^{3}H]Met-TRH binding to whole oocytes or oocyte membranes. As an alternative, we measured the time course for recuperation of the response after a second TRH addition following a first challenge with the hormone. Figure 4 shows that in oocytes injected with WT receptor cRNA, the response to a second addition of TRH at saturating concentration once 5 min of washout have elapsed, was still less than half of that obtained in the same oocytes along the first exposure to TRH. In contrast, a washout period of 2 min between the two TRH additions was enough to restore the response to the hormone in oocytes expressing Asp^{105} mutant receptors. This demonstrates that, at least in oocytes injected with Asp^{105} receptor cRNA, 2 min of washout is enough to dissociate the hormone from its receptor. Otherwise, it would be impossible to reproduce the initial response. On the other hand, the observed differences in recuperation time should not be due to components localized along the response cascade far beyond the receptor itself, since the responses obtained in oocytes expressing WT and mutant receptors were of the same magnitude and characteristics.

Restoration of the initial response in Asp^{105} mutant receptors demonstrates that TRH is able to fully interact again with a receptor emptied by the washout period. However, it could be possible that the WT reduced second response is not due to slower dissociation of hormone from the receptors, but to an enhanced period of desensitization, even though the hormone did not remain bound. This would preclude the establishment of any correlation between recovery kinetics and rates of ligand dissociation. Existence of desensitization of response for both WT and mutant receptors is demonstrated by decreases in current levels whilst the oocytes are being exposed to ligands. However, as shown in Figure 4 (panels B and C), careful comparison of response recovery times for TRH and Met-TRH indicates that, at least in a certain range, a correlation exists between ligand−receptor affinity (identified by EC_{50} values) and time of recovery. The longest recovery times corresponded to WT receptors activated with Met-TRH, whereas Asp^{105} mutant receptors showed the shortest recovery time in the presence of TRH. Intermediate and increasing recovery time values that followed the EC_{50} decrements were obtained for Asp^{105} mutant receptors activated with Met-TRH and WT receptors challenged with TRH, respectively. The scatter due to variability of individual oocytes and donors precluded a more quantitative comparison of recovery rates among different days, but similar results to those reproduced in Figure 4 were obtained in three additional experiments (see also Figure 6).

It is interesting that shortening of recovery times was observed up to a given level (panel C of Figure 4). Bigger displacements of affinity and/or faster hormone dissociation rates could be expected to cause even smaller recovery times. However, these were not detected probably due to desensitization of oocyte response elements with shorter washout times. In fact, response recovery times shorter than those obtained for TRH and Asp^{105} receptors were not detected with Asn^{105} or Glu^{105} mutants, even though they show lower affinities than those of Asp^{105} mutant TRH-Rs (not shown). Thus, it can be concluded that mutation of residue 105 not only modifies the affinity of TRH-R by its ligands, but also its ability to recover from a challenge with hormone. At least for a certain range, desensitization of oocyte response elements is not involved in such a variation. On the other hand, unless the nature of the ligand affects it, our results

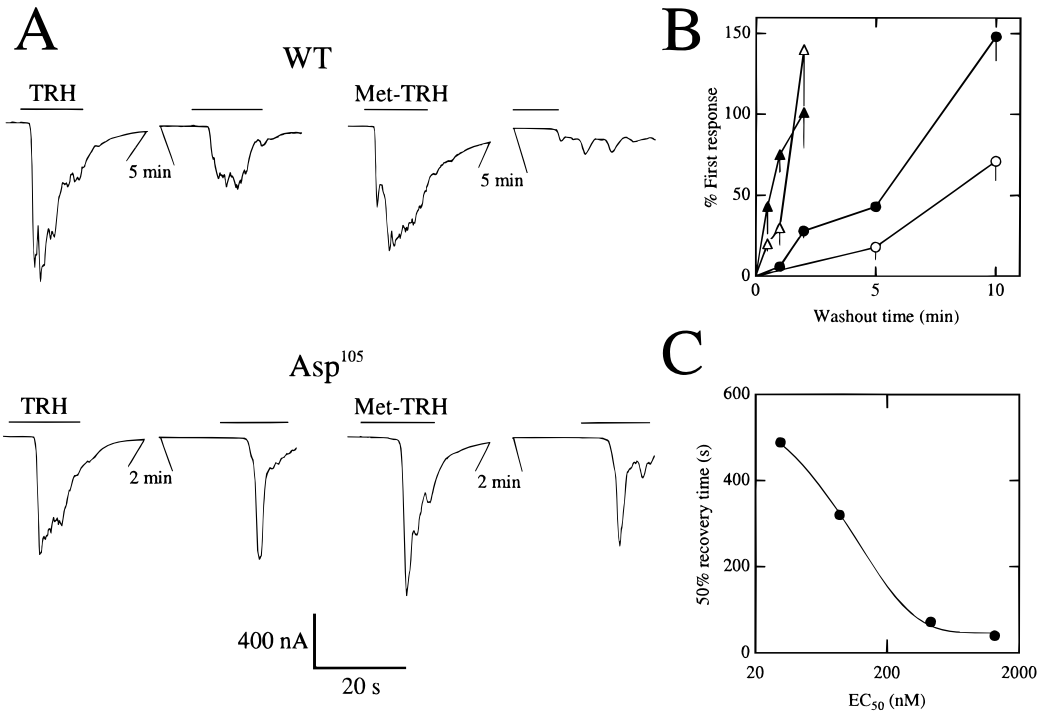

FIGURE 4: Recovery of TRH-induced responses in oocytes expressing wild-type (WT) or Asp^{105} mutant receptors after challenge with TRH or Met-TRH. (A) Responses to two successive additions of TRH or Met-TRH are shown. Traces have been interrupted when indicated. The times shown in the graph represent the total period elapsed between the end of the first and the beginning of the second addition of hormone. The duration of perfusion with saline plus hormone is indicated by horizontal lines above the traces. TRH concentrations were 0.5 μM for oocytes expressing WT receptors and 10 μM for oocytes injected with Asp^{105} mutant receptor cRNA. Concentrations of Met-TRH were 0.2 μM and 4.5 μM for WT and Asp^{105} mutant receptors, respectively. (B) Magnitude of the second response (expressed as percentage of the first one), as a function of time between two successive additions of hormone. Data are expressed as mean minus SEM. Initial maximum responses from three to four oocytes of the same donor have been averaged for every experimental point. The type of receptor expressed and the hormone used to induce responses are as follows: open circles, WT receptors and Met-TRH; closed circles, WT receptors and TRH; open triangles, Asp^{105} mutant receptors and Met-TRH; closed triangles, Asp^{105} mutant receptors and TRH. (C) Time necessary to recover 50% of the initial response in *B* is plotted against the EC_{50} value for every receptor and type of agonist used. EC_{50} values are from Figure 3. Fitting of the data to an exponential function is represented by a continuous line on the graph.

also suggest that enhanced ligand dissociation rates caused by alterations in residue 105 may contribute to lower affinities observed with mutant receptors.

Subsecond Stimulation of Oocytes Expressing Wild-Type TRH Receptors Mimics the Effect of Gln^{105} Mutations Shortening Oocyte Response Recovery Times. A corollary of the interpretations advanced above would be that WT receptors are converted during the first TRH addition to a form from which the hormone dissociates slowly. Such a conversion would be impaired in receptors mutated in residue 105. Providing that association rates of the receptor and hormone are rapid (Ramsdell & Tashjian, 1986), it could be expected that maximal responses can be induced with shorter times of exposure. Furthermore, it could be expected also that a rapid hormone dilution following such a short exposition would prevent the receptor from reaching the slow dissociation form. To test this hypothesis, we have developed a laminar flow chamber in which hormone is applied directly to the surface of the oocyte, followed by a rapid switch to hormone-free medium that rapidly eliminates the ligand from the oocyte proximity (see Materials and Methods). A small electronic valve is used to limit the application of hormone as desired, even in the subsecond time span. Figure 5 shows that applications of TRH by as short as 500 ms are enough to trigger responses in oocytes injected with WT receptor cRNA of the same magnitude as those developed along 3 s hormone applications. Increasing application times up to 20 s does not modify the levels of response obtained after 3 s of treatment. However, a second addition of TRH following a 3 s first hormone treatment and 2 min of washout induces a very small response (13% of the initial one). In contrast, the second response amounts 60% of the initial one after lowering the time of the first TRH exposure to 500 ms. Use of shorter application times would complicate the interpretation of the results due to reduction of oocyte responses. On the other hand, 1 s first additions of TRH produced intermediate results to those obtained after applications of 0.5 and 3 s (not shown).

A final validation of this technical approach to correlate recovery times with dissociation rates and/or affinities came from experiments in which oocytes from the same donor are used to compare the results of short additions of Met-TRH with those obtained with TRH. As shown in Figure 6, the response obtained during a second challenge with the hormone once 2 min of washout have elapsed, was 23% and 70% of the response to a first TRH addition of 3 s and 500 ms, respectively. However, the second response was significantly lower ($p < 0.05$) when Met-TRH instead of TRH was used under the same conditions. Thus, a second response that amounted only 9% of the first one was obtained after two successive 3 s additions of Met-TRH. On the other hand, the second response averaged 35% of the first one when an initial 500 ms treatment with Met-TRH was followed by a second challenge with the hormone for 3 s two minutes later. Similar results were obtained in two additional experiments using different batches of oocytes. These data indicate again that, under the conditions used, differences in the magnitude of the response are not involved

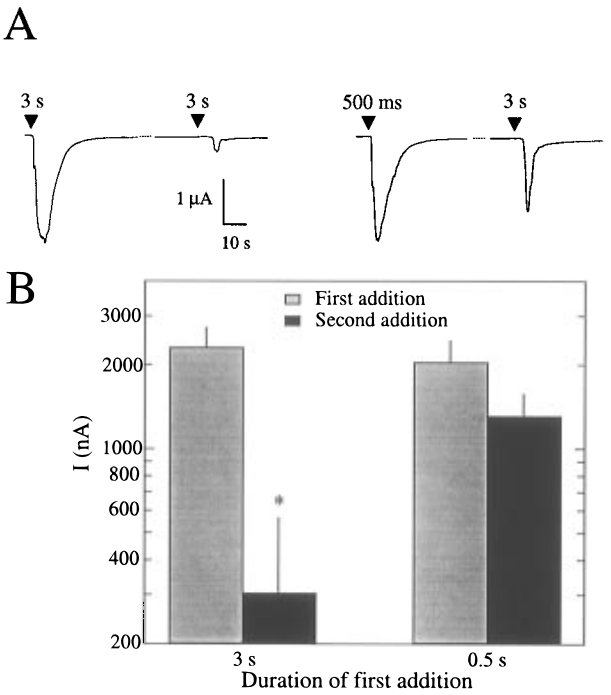

FIGURE 5: Recovery of responses induced by short expositions to TRH in oocytes expressing wild-type TRH-Rs. (A) Responses induced in two oocytes from the same donor along two successive additions of TRH separated by 2 min of washout. 1 μM TRH was applied when signaled by arrowheads for the time indicated above the traces. Dotted lines between both hormone additions represent periods at which the current recordings are not shown. (B) Averaged responses to two successive applications of 1 μM TRH. Application time during the first challenge with TRH is indicated on the abscissa. The second treatment with the hormone always lasted for 3 s. Averaged maximal responses plus 95% confidence intervals for 18 oocytes from three frogs are shown. Note the logarithmic scaling of the current data. Significant differences in response during the second addition *vs* the response magnitude reached along the first exposure to TRH are indicated with an asterisk ($p < 0.001$).

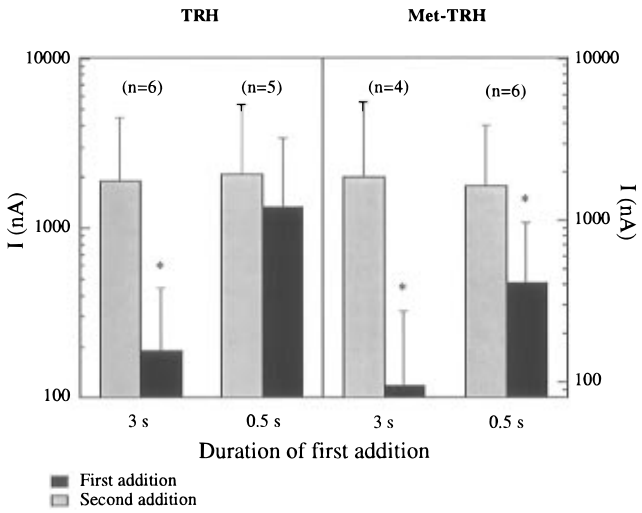

FIGURE 6: Difference in levels of response recovery after short expositions of oocytes expressing wild-type TRH-Rs to TRH or Met-TRH. Oocyte responses were measured along two successive additions of 1 μM TRH (left) or 400 nM Met-TRH (right) separated by 2 min of washout. The duration of the first application of hormone is indicated on the abscissa. The second treatment with hormone always lasted for 3 s. Averaged responses plus 95% confidence intervals are shown for the indicated number of oocytes from the same donor. Note the logarithmic scaling of the current data. Significant differences in response during the second addition *vs* the response magnitude reached along the first exposure to TRH are indicated with an asterisk ($p < 0.05$).

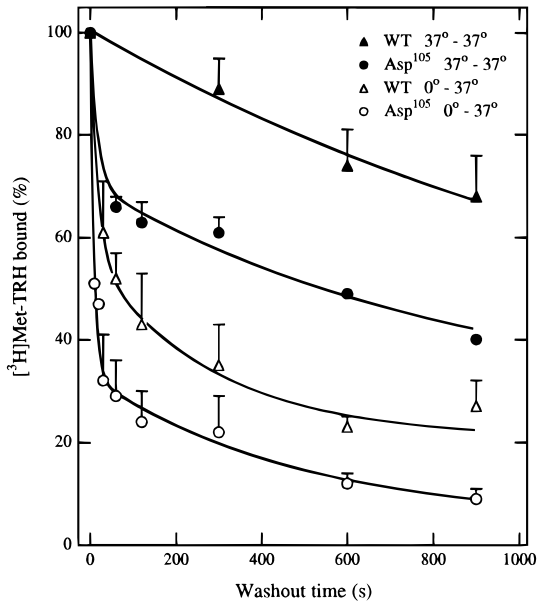

FIGURE 7: Effect of Gln 105 to Asp mutation in [^{3}H]Met-TRH dissociation kinetics. Dishes of transfected cells were incubated with [^{3}H]Met-TRH for 2 h at 0 or 37 °C as described in Methods. The cells were then washed and the amount of [^{3}H]Met-TRH bound to cells was determined after the times shown on the abscissa at 37 °C. The type of transfected receptor and the temperatures used along the binding and dissociation periods are indicated on the right of the symbols. Averaged values plus SEM from two experiments performed with triplicate samples are shown.

in the different recuperation levels. On the other hand, they demonstrate that a correlation can be established also in WT receptors between ligand affinity and response recovery time providing that hormone and receptor contact time is carefully chosen. Furthermore, they also suggest that decreased recovery times are caused, at least in part, by enhanced rates of ligand dissociation.

Direct Demonstration of Enhanced Ligand Dissociation Rates in COS-7 Cells Transfected with Residue 105 Mutant TRH Receptors. To demonstrate in a direct way that modifications of residue 105 cause enhanced rates of ligand dissociation from TRH receptors, the kinetics of [^{3}H]Met-TRH dissociation was studied in cultures of COS-7 cells transfected with WT or Asp^{105} mutant receptors. Measurements of [^{3}H]Met-TRH specific binding and subsequent dissociation of labeled ligand were not feasible with Asn^{105} or Glu^{105} receptor-transfected cells due to the lower affinities found with these mutant receptors. It has been previously shown that the dissociation rate of TRH from its receptor is strikingly dependent of the temperature at which the binding reaction is performed (Hinkle & Kinsella, 1982). Subsequently, [^{3}H]Met-TRH was bound to transfected cells at 37 or 0 °C for 2 h, and the cell monolayers were washed to remove free hormone. The rates of dissociation were then compared for WT and Asp^{105} receptors measuring the amount of labeled ligand remaining bound after maintaining the cells for different periods at 37 °C. As shown in Figure 7, when binding to cells expressing WT receptors was performed at 37 °C, 25 min were necessary to dissociate half of the [^{3}H]-Met-TRH specifically bound to cells. Thus, 68% of [^{3}H]-Met-TRH still remained bound 15 min after removal of ligand from the medium. Consistent with previous results in GH cells (Hinkle & Kinsella, 1982), 50% of labeled ligand dissociated in around 1 min when the binding reaction was carried out at 0 °C, and only 20−25% of [^{3}H]Met-TRH remained bound after 15 min of dissociation reaction. In

contrast to these results, the rate of dissociation was clearly accelerated in cells expressing Asp^{105} mutant receptors, regardless of performance of the binding reaction at 37 or 0 °C. Thus, only 8 min and around 10 s were enough to dissociate half of the [^{3}H]Met-TRH at the two preincubation temperatures. Subsequently, when the dissociation was continued for 15 min, only 40% and 9% of specifically bound [^{3}H]Met-TRH remained associated with cells to which the ligand was previously bound at 37 or 0 °C, respectively.

DISCUSSION

The critical role of an acidic residue in TM-III of G protein-coupled receptors for biogenic amine ligands is well established (Strosberg, 1991; Dohlman et al., 1991; Ostrowski et al., 1992; Savarese & Fraser, 1992; Wess, 1993). However, little is known about the importance of residues located in an equivalent position of receptors for small uncharged ligands such as TRH. Introduction of a negative charge in this position is not enough for activation of the TRH-R by several biogenic amines. This indicates that, besides the ion−ion interaction between the positively charged amino head group of these ligands and the receptor acidic residue, additional interactions dictate the binding and/or activation of different receptors by their ligands. These interactions are not present between the TRH-R and the tested biogenic amines. Lack of a negatively charged residue in most receptors for ligands different from biogenic amines can be interpreted as simply due to a negative selection against positioning a charged residue in a hydrophobic domain. Given the nearly normal functionality of Asp^{105} and Asp^{110} mutant TRH-Rs, such an interpretation seems unlikely. Alternatively, it could be assumed that the nature of the residue in that position is important for a correct interaction of the ligand with the receptor and/or its subsequent activation. In support to this view, it has been proposed that the Asp residue in TM-III of muscarinic acetylcholine receptors is even more important for ternary hormone−receptor−G protein complex formation, and receptor activation, than for binary hormone−receptor complex formation, and binding (Hulme et al., 1993).

Our results indicate that modifications of the side chain of Gln^{105} in the TRH-R have important effects on its functionality. Elimination of the amido group drastically reduces the potency of oocytes expressing mutant receptors to respond to TRH whereas the maximal level of response is not altered by the mutation. Charge effects do not seem involved in the observed effects, since neither Gln^{105} to Asp nor Asn^{110} to Asp mutants are similarly altered. To discard that the presence of the negative charge is the cause of the lower potency found with Glu^{105} receptors and that the improvement obtained in the Asp^{105} mutant is due to the longer distance between this charge and other groups, due to the smaller volume of the side chain of Asp as compared to that of Glu, residue 105 was also mutated to Asn. As with Asp, a side chain smaller than that of Glu is contributed by Asn, but the negative charge is not present in Asn^{105} receptors. However, no potency improvement respect to Asp^{105} is obtained with Asn^{105} and, in fact, a significantly lower potency is observed. Altogether, this suggests that the amido group in Gln^{105} plays an important role in the receptor and that the modifications on this group are the cause of the reduced ability of the mutant receptors to respond to TRH. On the other hand, since Asn^{105} mutant receptors show a 160- and 40-fold increase in EC_{50} values for TRH and Met-TRH, respectively, as compared with WT receptors, the correct positioning of the amido group is an important determinant of its role on the receptor. Such an important contribution of the Gln^{105} hydrogen bond−donor amido group to receptor functionality seems challenged by the small EC_{50} increase observed in oocytes expressing Asp^{105} mutant receptors that also lack such an amido group. We hypothesize that, since Asp contributes a side chain smaller than those of Glu and Asn, it is possible that loss of hydrogen bonding interactions is partially compensated by a water molecule. A similar mechanism has been evoked in tyrosyl-tRNA synthetase and lysozyme to explain improved substrate binding after moving a group too far as to continue forming a hydrogen bond (Ferst et al., 1985; Ward et al., 1990). Our interpretation would be also consistent with the almost normal EC_{50} value observed with Ser^{105} mutant receptors in which a small side chain is introduced in position 105, and with the very small decrease in affinity previously noted with Ala^{105} mutant receptors (Perlman et al., 1994a). Furthermore, it could explain that substitutions with residues as large as Leu or Tyr eliminate receptor functionality. Unfortunately, neither TRH-R specific antibodies nor other means to detect inactive receptors are available, and lack of expression of these receptors in the membrane cannot be excluded. It is important to note also that the exact location of single amino acid residues within the membrane remains unknown. Subsequently, although our results are consistent with the proposed hypothesis, we are currently unable to provide further evidence to support it.

Regardless of the specific structural reorganizations caused by the mutations, our data are consistent with the idea that at least some substitutions of residue 105 do not cause a gross disruptive alteration of receptor structure. First, in most cases, mutation replaces an existing side chain with a smaller side chain, a strategy that minimizes the probability of changes in protein structure (Ward et al., 1990). Exceptions to this rule can be substitutions of Gln^{105} by Tyr or Lys. Second, comparable levels of WT and Glu^{105}, Asn^{105}, Asp^{105}, and Ser^{105} mutant receptors are expressed at the cell surface, since maximal levels of oocyte response are dependent on TRH-R number (Straub et al., 1989; Matus-Leibovitch et al., 1994) and binding analysis of Asp^{105} mutant receptors expressed in transfected COS-7 cells indicates that the number of mutant receptors is slightly bigger than that of WT receptors (de la Peña and Barros, unpublished data). Third, at least Glu^{105}, Asn^{105}, Asp^{105}, and Ser^{105} mutant receptors are able to adopt functional resting and active conformations, since they can effectively activate phosphatidylinositol turnover and oocyte Cl^- currents in response to TRH and Met-TRH binding.

Recent studies involving extensive mutagenesis of residues located in different domains of the TRH receptor, including Gln^{105}, and computer modeling of the hormone and receptor structures, allowed the elaboration of two different models for the receptor binding pocket and for the participation of different receptor amino acids in ligand−receptor interactions. In one case, TRH would bind entirely within the transmembrane helices and a direct interaction would be established between the hormone and residues 106 and 110 in TM-III. Gln^{105} would not interact directly with TRH. However, interaction between the side chain C═O of Gln^{105} and Arg^{306} in transmembrane helix VII would lead to indirect perturbations in the binding pocket when Gln^{105} is modified

(Perlman et al., 1994a, 1995). As an alternative, a second model has been proposed in which binding of TRH involves mainly extracellular receptor domains. In this case, TM-III residues including those at positions 106 and 110 would affect TRH binding indirectly due to changes in overall receptor structure (Han & Tashjian, 1995a,b). Our results suggest that modifications of Gln^{105} cause an indirect alteration in the ligand binding pocket. Parallel impairment of interactions of the receptor not only with agonists, but also with the competitive TRH antagonist CDPX favours this interpretation. As indicated above, Tyr^{106} has been implicated in a direct Tyr^{106}−TRH interaction. Alterations in receptor functionality by mutations in Gln^{105} does not seem to be simply due to changes in location of a primary hormone docking point contributed by Tyr^{106}, since an apparently additive loss of potency is observed by mutating both 105 and 106 residues. The establishment of an H-bond between the side chain C═O of Gln^{105} and the side chain of Arg^{306} (Perlman et al., 1994a, 1995) is unlikely, since receptor function is severely impaired in Glu^{105} mutant receptors that maintain the side chain C═O of Gln^{105}. In our study, the extent of receptor function impairment after mutating Gln^{105} to Glu clearly differs from that previously reported (Perlman et al., 1994a). We do not presently know the reasons for this discrepancy.

It is important to note that our data do not allow us to clearly discriminate between any of the two proposed models for the TRH binding pocket. However, it is tempting to speculate that residue 105 substitutions cause a reduced potency for ligands indirectly favoring a naturally occurring conformation of the receptor that binds them with low affinity (Nardone & Hogan, 1994). In this case, ligand binding to WT receptors would drive them to the high-affinity state. Since probably there is a rapid conversion of the initial receptor conformation to other conformations after ligand binding, it would be difficult to detect it experimentally in the low-affinity state. In residue 105 mutant receptors, state transitions are slow and it could be easier to detect both low- and high-affinity forms.

Lower affinities [identified by EC_{50} value shifts for systems of comparable efficacy; see Limbird (1986), Perlman et al. (1994b, 1995), and Han and Tashjian, (1995a,b)] in receptors mutated at residue 105 could reflect either decreases in association or increases in dissociation kinetic rates. Comparison of these parameters between WT and mutant TRH-Rs using binding experiments is strongly hampered by the reductions in affinity caused by the mutations. Although indirect, an alternative approach is provided by studies of response parameters in oocytes expressing the receptors. The high sensitivity and excellent temporal resolution extending even to the subsecond time span of the *Xenopus* oocyte system makes it an invaluable tool to investigate some of the mechanisms of signal transduction initiated by ligand binding. We have used this system to gain further insights about the functional consequences of alterations in residue 105 of the TRH-R. The similar latency periods between hormone addition and the onset of responses in oocytes expressing either WT or residue 105 mutant receptors could be taken as an indication that association rates are not lengthened by the mutations. However, since the latency period is a consequence of a step after formation of TRH−receptor complexes (Straub et al., 1989), only association rate increments large enough to make the hormone−receptor interaction rate-limiting would cause a variation in the latency of the response. Therefore, a contribution of small increments in association rate to lower binding affinities cannot be excluded. Nevertheless, our results demonstrate both in oocytes and transfected cells a clear effect of Gln^{105} mutation in dissociation rates.

The reduced responses to a second addition of hormone obtained in oocytes expressing TRH-Rs are not due to differences in oocyte response desensitization since the initial responses of oocytes expressing WT and mutant receptors were always of the same magnitude and characteristics. However, very different recovery times were obtained in both groups of oocytes. This indicates that, at least for recuperation times bigger than those of Asp^{105} mutant TRH-Rs, the longer times necessary to recover the initial responses are due to differences in the receptor itself or in a component of the signalling cascade closely related to it.

The fast restoration of the initial response in the case of Asp^{105} receptors demonstrates that the short washout times used are enough to dissociate the hormone from the mutant receptors. Otherwise, it would be impossible to reproduce the initial response by reintroducing the ligand in the chamber. On the other hand, the longer times necessary to recover the response with WT receptors should be due either to a slower ligand dissociation rate or to an increased period of desensitization at (or close to) the receptor level. Challenge of oocytes carrying WT or Asp^{105} TRH-Rs with TRH or Met-TRH demonstrates a correlation between ligand−receptor affinity and time for response recovery. Furthermore, the effects of mutating Gln^{105} on response recovery times are mimicked in oocytes expressing WT receptors by reducing TRH application times from 3 s to less than 1 s without modifying the levels of response. These results suggest that mutations of residue 105 enhance ligand dissociation rates. A direct demonstration of this enhancement has been obtained by comparison of [^{3}H]Met-TRH dissociation in COS-7 cells transfected with either WT or Asp^{105} mutant TRH-Rs.

It is important to note that our results do not exclude the possibility of multiple active receptor conformations induced by the ligand and that the conformation(s) leading to an enhanced ligand dissociation is paralleled by a conformation in which receptor desensitization is also modified. Thus, shorter periods of ligand−receptor contact as a result of increased dissociation rates could cause lower desensitization levels by mechanisms such as phosphorylation by a receptor kinase that uses the ligand−receptor complex as substrate. Interestingly, very rapid desensitization of oocyte responses induced by TRH occurring before calcium has gone up or TRH has dissociated has been reported (Lipinsky et al., 1995). If this or another mechanism contribute also to differences in oocyte response observed after alteration of residue 105 remains to be established. In any case, this would emphasize further the key role of position 105 in TM-III not only maintaining a proper structure of the ligand−receptor interaction domain, but also modulating receptor conformations in areas involved in such a desensitization, presumably located in the cytoplasmic domains.

Previous studies in GH_3 cells indicate that the initial actions of TRH occur through a form of the receptor with rapid dissociation kinetics. Subsequently, the hormone−receptor complex converts in a temperature-dependent fashion to a form with slow dissociation kinetics (Hinkle & Kinsella, 1982; Ramsdell & Tashjian, 1986). The results presented here suggest that WT receptors are converted

during the first TRH addition to a form from which the hormone dissociates slowly. Such a shift would be impaired in receptors mutated in residue 105. It has been proposed that this shift is important for full activation of receptors and generation of a maximal biological response (Ramsdell & Tashjian, 1986). This opens the possibility that, apart from their involvement in regulation of hormone−receptor interactions, residues located in TM-III positions equivalent to those of the counterions for biogenic amines can also play a role, directly or indirectly, in receptor conformational changes leading to receptor activation, and hence to signal transduction. An indirect support to this hypothesis is provided by the fact that, although the three-dimensional structure of the TRH-R and the precise location of single amino acid residues within the membrane are currently unknown, TM-III probably occupies a central position more deeply buried in the structure of the G protein-coupled receptors (Baldwin, 1993).

ACKNOWLEDGMENT

We thank Dr. C. L. Otín for critical reading of the manuscript and Dr. J. Lerma for advice on construction of the fast application chamber.

REFERENCES

Baldwin, J. M. (1993) *EMBO J. 12*, 1693−1703.
Battey, J. F., Way, J. M., Corjay, M. H., Shapira, H., Kusano, K., Harkins, R., Wu, J. M., Slattery, T., Mann, E., & Feldman, R. I. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 395−399.
Bonner, T. I., Buckley, N. J., Young, A. C., & Brann, M. R. (1987) *Science 237*, 527−532.
Bunzow, J. R., Van Tol, H. H. M., Grandy, D. K., Albert, P., Salon, J., Christie, M., Machida, C. A., Neve, K. A., & Civelli, O. (1988) *Nature 336*, 783−787.
Chung, F.-Z., Wang, C. D., Potter, P. C., Venter, J. C., & Fraser, C. M. (1988) *J. Biol. Chem. 263*, 4052−4055.
Cotecchia, S., Schwinn, D. A., Randall, R. R., Lefkowitz, R. J., Caron, M. G., & Kobilka, B. K. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 7159−7163.
de la Peña, P., Delgado, L. M., del Camino, D., & Barros, F. (1992a) *Biochem. J. 284*, 891−899.
de la Peña, P., Delgado, L. M., del Camino, D., & Barros, F. (1992b) *J. Biol. Chem. 267*, 25703−25708.
de la Peña, P., del Camino, D., Pardo, L. A., Domínguez, P., & Barros, F. (1995) *J. Biol. Chem. 270*, 3554−3559.
Dohlman, H. G., Thorner, J., Caron, M. G., & Lefkowitz, R. J. (1991) *Annu. Rev. Biochem. 60*, 653−688.
Evans, C. J., Keith, D. E. Jr., Morrison, H., Magendzo, K., & Edwards, R. H. (1992) *Science 258*, 1952−1955.
Fersht, A. R., Shi, J.-P., Knill-Jones, J., Lowe, D. M., Wilkinson, A. J., Blow, D. M., Brick, P., Carter, P., Waye, M. M. Y., & Winter, G. (1985) *Nature 314*, 235−238.
Gantz, I., Schäffer, M., del Valle, J., Logsdon, C., Campbell, V., Uhler, M., & Yamada, T. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 429−433.
Gaylinn, B. D., Harrison, J. K., Zysk, J. R., Lyons, C. E., Lynch, K. R., & Thorner, M. O. (1993) *Mol. Endocrinol. 7*, 77−84.
Gershengorn, M. C., Heinflink, M., Nussenzveig, D. R., Hinkle, P. M., & Falck-Pedersen, E. (1994) *J. Biol. Chem. 269*, 6779−6783.
Han, B., & Tashjian, A. H., Jr. (1995a) *Mol. Endocrinol. 9*, 1708−1719.
Han, B., & Tashjian, A. H., Jr. (1995b) *Biochemistry 34*, 13412−13422.
Hinkle, P. M. (1989) *Ann. N.Y. Acad. Sci. 553*, 176−187.
Hinkle, P. M., & Kinsella, P. A. (1982) *J. Biol. Chem. 257*, 5462−5470.
Hulme, E. C., Curtis, C. A. M., Page, K. M., & Jones, P. G. (1993) *Cell. Signalling 5*, 687−694.
Julius, D., MacDermott, A. B., Axel, R., & Jessell, T. M. (1988) *Science 241*, 558−564.
Kimura, T., Tanizawa, O., Mori, K., Brownstein, M. J., & Okayama, H. (1992) *Nature 356*, 526−529.
Leong, S. R., Kabakoff, R. C., & Hébert, C. A. (1994) *J. Biol. Chem. 269*, 19343−19348.
Limbird, L. E. (1986) *Cell Surface Receptors: A Short Course on Theory and Methods*, Martinus Nijhoff Publishing, Boston, MA.
Lin, H. Y., Harris, T. L., Flannery, M. S., Aruffo, A., Kaji, E. H., Gorn, A., Kolakowski, L. F., Jr., Lodish, H. F., & Goldring, S. R. (1991) *Science 254*, 1022−1024.
Lipinsky, D., Nussenzveig, D. R., Gershengorn, M. C., & Oron, Y. (1995) *Pflügers Arch. 429*, 419−425.
Machida, C. A., Bunzow, J. R., Searles, R. P., Van Tol, H., Tester, B., Neve, K. A., Teal, P., Nipper, V., & Civelli, O. (1990) *J. Biol. Chem. 265*, 12960−12965.
Matus-Leibovitch, N., Nussenzveig, D. R., Gershengorn, M. C., & Oron, Y. (1994) *Biochem. J. 303*, 129−134.
Morel, A., O'Carrol, A-M., Brownstein, M. J., & Lolait, S. J. (1992) *Nature 356*, 523−526.
Mounjoy, K. G., Robbins, L. S., Mortrud, M. T., & Cone, R. D. (1992) *Science 257*, 1248−1251.
Nardone, J., & Hogan, P. G. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 4417−4421.
Ostrowski, J., Kjelsberg, M. A., Caron, M. G., & Lefkowitz, R. J. (1992) *Annu. Rev. Pharmacol. Toxicol. 32*, 167−183.
Perlman, J. H., Laakonen, L., Osman, R., & Gershengorn, M. C. (1994a) *J. Biol. Chem. 269*, 23383−23386.
Perlman, J. H., Thaw, C. N., Laakonen, L., Bowers, C. Y., Osman, R., & Gershengorn, M. C. (1994b) *J. Biol. Chem. 269*, 1610−1613.
Perlman, J. H., Laakonen, L., Osman, R., & Gershengorn, M. C. (1995) *Mol. Pharmacol. 47*, 480−484.
Ramsdell, J. S., & Tashjian, A. H., Jr. (1985) *Mol. Cell. Endocrinol. 43*, 173−180.
Ramsdell, J. S., & Tashjian, A. H., Jr. (1986) *J. Biol. Chem. 261*, 5301−5306.
Saunders, J., & Freeman, S. B. (1989) *Trends Pharmacol. Sci.* (December Suppl.), 70−75.
Savarese, T. M., & Fraser, C. M. (1992) *Biochem. J. 283*, 1−19.
Strader, C. D., Sigal, I. S., Candelore, M. R., Rands, E., Hill, W. S., & Dixon, R. A.F. (1988) *J. Biol. Chem. 263*, 10276−10271.
Strader, C. D., Candelore, M. R., Hill, W. S., Dixon, R. A. F., & Sigal, I. S. (1989) *J. Biol. Chem. 264*, 16470−16477.
Strader, C. D., Gaffney, T., Sugg, E. E., Candelore, M. R., Keys, R., Patchett, A. A., & Dixon, R. A. F. (1991) *J. Biol. Chem. 266*, 5−8.
Straub, R. E., Frech, G. C., Joho, R. H., & Gershengorn, M. C. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 9514−9518.
Straub, R. E., Oron, Y., Gillo, B., Thomson, R., & Gershengorn, M. C. (1989) *Mol. Endocrinol. 3*, 907−914.
Strosberg, A. D. (1991) *Eur. J. Biochem. 196*, 1−10.
Thomas, K. M., Pyun, H. Y., & Navarro, J. (1990) *J. Biol. Chem. 265*, 20061−20064.
Vu, T.-K., Hung, D. T., Wheaton, V. I., & Coughlin, S. R. (1991) *Cell 64*, 1057−1068.
Ward, W. H. J., Timms, D., & Fersht, A. R. (1990) *Trends Pharmacol. Sci. 11*, 280−284.
Wess, J. (1993) *Trends Pharmacol. Sci. 14*, 308−313.
Yamada, Y., Post, S. R., Wang, K., Tager, H. S., Bell, G. I., & Seing, S. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 251−255.
Yokota, Y., Sasai, Y., Tanaka, K., Fujiwara, T., Tsuchida, K., Shigemoto, R., Kakizuka, A., Ohkubo, H., & Nakanishi, S. (1989) *J. Biol. Chem. 264*, 17649−17652.
Zhao, D., Yang, J., Jones, K. E., Gerald, C., Suzuki, Y., Hogan, P. G., Chin, W. W., & Tashjian, A. H., Jr. (1992) *Endocrinology 130*, 3529−3536.

BI9622534