A conserved Asn in TM7 of the thyrotropin receptor is a common requirement for activation by both mutations and its natural agonist

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Abstract The wide spectrum of naturally occurring mutations able to activate the thyrotropin (TSH) receptor provides a useful tool to approach the structure of the active state(s) of the glycoprotein hormone receptors. Here we show that the side-chain of the highly conserved N*7.49* (Asn 674) in TM7 is mandatory for activation of the TSH receptor, not only by TSH, but also by a panel of eight natural and two artificial activating mutations. Basal activity levels of the mutants were significantly decreased by suppression of the side-chain of N*7.49* (N*7.49*A double mutants). In addition, comparative effects of the N*7.49*A substitution on the ten mutants demonstrate that basal activity and agonist- or mutation-stimulated activity might involve different structural changes.

Key words: G protein-coupled receptor; Thyroid-stimulating hormone receptor; Constitutive activity; Natural mutation; Activation mechanism

1. Introduction

Glycoprotein hormone receptors (thyroid-stimulating hormone receptor (TSHr), luteinizing hormone/CG receptor (LH/CGr), follicle-stimulating receptor (FSHr)) constitute a subfamily of the well-conserved rhodopsin-like G protein-coupled receptors (GPCRs) [1]. They are characterized by a dual structure made of a large amino-terminal extracellular domain (ECD), the high-affinity binding site of the individual hormones [2–5], and a transmembrane region of seven α-helices (TMs), responsible for signal transduction. Binding of the hormones to the ECD triggers conformational changes of the seven TMs leading to an active state of the receptor. The glycoprotein hormone receptors are mainly coupled to Gs but, at high concentration of their agonists, they also couple to Gq [6]. The intramolecular mechanisms by which binding of the hormones to the ECD activates the transmembrane part of the receptors are the subject of active investigations but remain largely unknown.

In contrast to the LH/CGr and FSHr, the TSHr has been shown to present significant basal activity in the absence of ligand [7,8]. In addition, more than 25 naturally occurring mutations have been found to increase dramatically TSHr constitutive activity, leading to acquired diseases such as toxic adenomas or (rarely) thyroid cancer, and autosomal dominant forms of hereditary hyperthyroidism [9,10]. Quite unexpectedly, these mutations have been located in all places of the receptor: the ECD, the extracellular and intracellular loops, or the TM region. Interestingly, such activating mutations are less frequently found in the LH/CGr and the spectrum of residues involved seems to be narrower [11]. For the FSHr, no convincing natural mutant has been identified and the receptor is resistant to activation by many amino acid substitutions with a strong activating effect, when present in homologous segments of the TSH or LH/CG receptors [12].

In a recent study, we showed that a highly conserved Asn in TM7 (part of the canonical NPXXY signature of rhodopsin-like GPCRs) was implicated in the activation mechanism of the TSHr [13]. In the basal state of the receptor this residue N*7.49*^{674} (see Section 2 for description of the numbering system) was found to interact with a conserved Asp in TM6 (D*6.44*^{633}). Breaking of this interaction (e.g. as a consequence of natural or artificial mutations affecting D*6.44*^{633}) was proposed to release N*7.49*, rendering its side-chain available for interactions implicated in the activation mechanism. In agreement with this view, we showed that the presence of an Asn (or Asp) residue in position *7.49* was mandatory for activation of the receptor by TSH. Although it bound TSH with normal affinity, an N*7.49*A mutant lost the ability to be stimulated by TSH. The wide spectrum of mutations capable of activating the TSHr provides, in theory, a unique opportunity to approach the structure of the active state(s) of the glycoprotein hormone receptors. In the present study, as a first step in this direction, we demonstrate that the side-chain of N*7.49* was required for activation of the TSHr, not only by TSH, but also by a panel of eight natural and two artificial activating mutations.

2. Materials and methods

2.1. Numbering scheme of GPCRs

The standardized numbering system of Ballesteros and Weinstein

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Abbreviations: TSH, thyroid-stimulating hormone; GPCR, G protein-coupled receptor; TM, transmembrane helix; FACS, fluorescence activating cell sorting

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was used throughout to identify residues in the transmembrane segments of different receptors [14]. Each residue is identified by two numbers: the first (1 through 7) corresponds to the helix in which it is located; the second indicates its position relative to the most-conserved residue in that helix, arbitrarily assigned to 50. For instance N*7.49* is the asparagine in TM7, located one residue before the highly conserved proline P*7.50*. Residue D*2.50* corresponds to Asp 460 in the TSHr numbering; S*3.36* to Ser 505; E*3.49* to Glu 518; V*5.58* to Val 597; T*6.43* to Thr 632; D*6.44* to Asp 633 and N*7.49* to Asn 674. For residues located outside the TMs (S281 in the ECD and I486 in the first extracellular loop, Y601 and D*6.30* at the border between TMs and intracellular loops), we used the numbering system of the TSHr.

2.2. Site-directed mutagenesis of the TSH receptor

Plasmids encoding the various TSHr mutants were constructed by site-directed mutagenesis using PCR amplifications as described previously [13]. Amplified fragments containing the mutations were cloned after digestion with appropriate restriction enzymes into the pSVL expression vector (Amersham Pharmacia Biotech, Freiburg, Germany) containing the wild-type TSHr [15] using standard procedures. All PCR-generated receptor fragments were verified by sequencing before transfection.

2.3. Transfection and assays

COS-7 cells were grown in Dulbecco's modified Eagle's medium supplemented with fetal bovine serum 10%, sodium pyruvate 1 mM, penicillin 100 IU/ml, streptomycin 100 μg/ml and fungizone 2.5 μg/ml. Cells were seeded at a density of 350 000 cells/3 cm dish. One day later, they were transfected (500 ng DNA/dish) by the DEAE–dextran method followed by a dimethylsulfoxide shock [16]. Two days after transfection, cells were used for flow immunocytofluorometry and cAMP studies. Duplicate dishes were used for each assay. Each experiment was repeated at least three times. Cells transfected with pSVL alone were always run as controls.

2.4. Flow immunocytofluorometry

Experiments were performed as described previously [13]. Briefly, cells were detached with PBS–EDTA–EGTA and after centrifugation and removing of the supernatant, incubated with the BA8 monoclonal antibody, obtained from genetic immunization with the wild-type TSH receptor cDNA [17]. The cells were washed once and incubated with fluorescein-conjugated gamma chain-specific goat anti-mouse IgG (Sigma, St. Louis, MO, USA). Propidium iodide was used for detection of damaged cells, which were excluded from the analysis. Cells were washed once again and resuspended in 250 μl PBS–BSA 0.1%. The fluorescence of 30 000 cells per tube was assayed by a FACScan flow cytofluorometer (Beckton Dickinson, Erembodegem, Belgium).

2.5. cAMP determination

Cells were washed with Krebs–Ringer–HEPES buffer (KRH isotonic, pH 7.4). After a preincubation in KRH at 37°C for 30 min, cells were incubated in the same buffer supplemented with Rolipram 25 μM (a cAMP phosphodiesterase inhibitor, gift from the Laboratory of J. Logeais, France), in the absence or presence of various bTSH concentrations (Sigma, St. Louis, MO, USA). One hour later, the medium was removed and HCl 0.1 M was added to the cells. The cellular extracts were dried overnight in a vacuum concentrator (Savant) and intracellular cAMP was determined exactly as described previously [18]. Basal cAMP was normalized to cell-surface expression for each of the constructs. To this end, specific cAMP accumulation (= cAMP of receptor-transfected cells − cAMP of the pSVL-transfected cells) is divided by the specific fluorescence activating cell sorting (FACS) value (= fluorescence of receptor-transfected cells − fluorescence of pSVL-transfected cells), which can be summarized as: specific basal activity = (cAMP(receptor) − cAMP(pSVL))/(FACS(receptor) − FACS(pSVL)). The values are then expressed as percentage of specific basal activity of the wild-type TSHr. The production of cAMP in response to TSH, within the cells which have effectively been transfected, was estimated and expressed as 'fold stimulation' in the following way: fold stimulation of cAMP accumulation by TSH = ($cAMP_{+TSH}$(receptor) − [($cAMP_{+TSH}$(pSVL) × (1-transfection efficiency)])/($cAMP_{basal}$(receptor) − [($cAMP_{basal}$(pSVL) × (1-transfection efficiency)]).

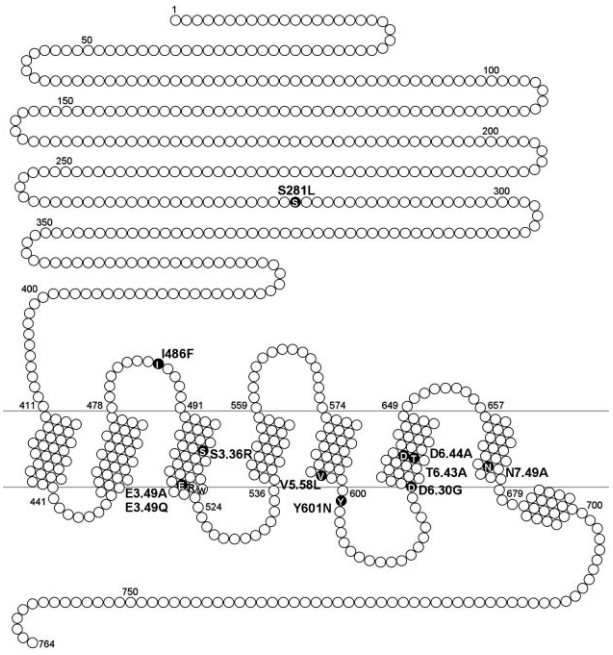

Fig. 1. Schematic representation of the TSHr with indication of the mutations studied. Mutated residues are shown in white inside black circles with the names of the mutants indicated. See Section 2 for description of the numbering system.

3. Results and discussion

3.1. A common 'conformational pathway' in all activating mutations of the TSHr

Amongst the numerous naturally occurring mutations that have been shown to increase the basal activity displayed by the wild-type TSHr [7,8,10,19], a subset of eight amino acid substitutions were selected for their diverse locations in the receptor structure (see Fig. 1): in the ECD (S281L), in the extracellular loops (I486F), in the TMs (S*3.36*^{505}R, V*5.58*^{597}L, T*6.43*^{632}A, D*6.44*^{633}A), or at the border between TMs and intracellular loops (Y601N, D*6.30*G). Despite their being widely dispersed all these natural mutants are known to induce conformational changes leading to increased constitutive activity [9,16] (see Fig. 2). In addition we constructed two artificial mutations affecting the Glu residue in position *3.49* immediately upstream of the most-conserved residue in rhodopsin-like GPCRs (R*3.50* of the E/DRY motif). Information available from other GPCRs predicts that the E*3.49*A and E*3.49*Q mutants would display increased constitutive activity [20–22], and this was indeed the case (Fig. 2).

These ten gain-of-function TSHr mutations were used to explore whether the highly conserved Asn residue in position *7.49* (the N of the NPXXY motif), which we found to be mandatory for activation of the receptor by TSH [13], was similarly implicated in the active conformation(s) achieved by the individual mutants. In previous experiments, we showed that, in the basal state, Asn *7.49* interacted with D*6.44,* a conserved residue in TM6 of the glycoprotein hormone receptors. Disruption of this interaction, by mutation of D*6.44* or secondary to stimulation by TSH, was proposed to release the side-chain of N*7.49*, allowing it to engage in interactions involved in stabilizing the active state(s) of the receptor [13]. We wanted to investigate here the effect of the N*7.49*A substitution when it is added on the background of each of the ten

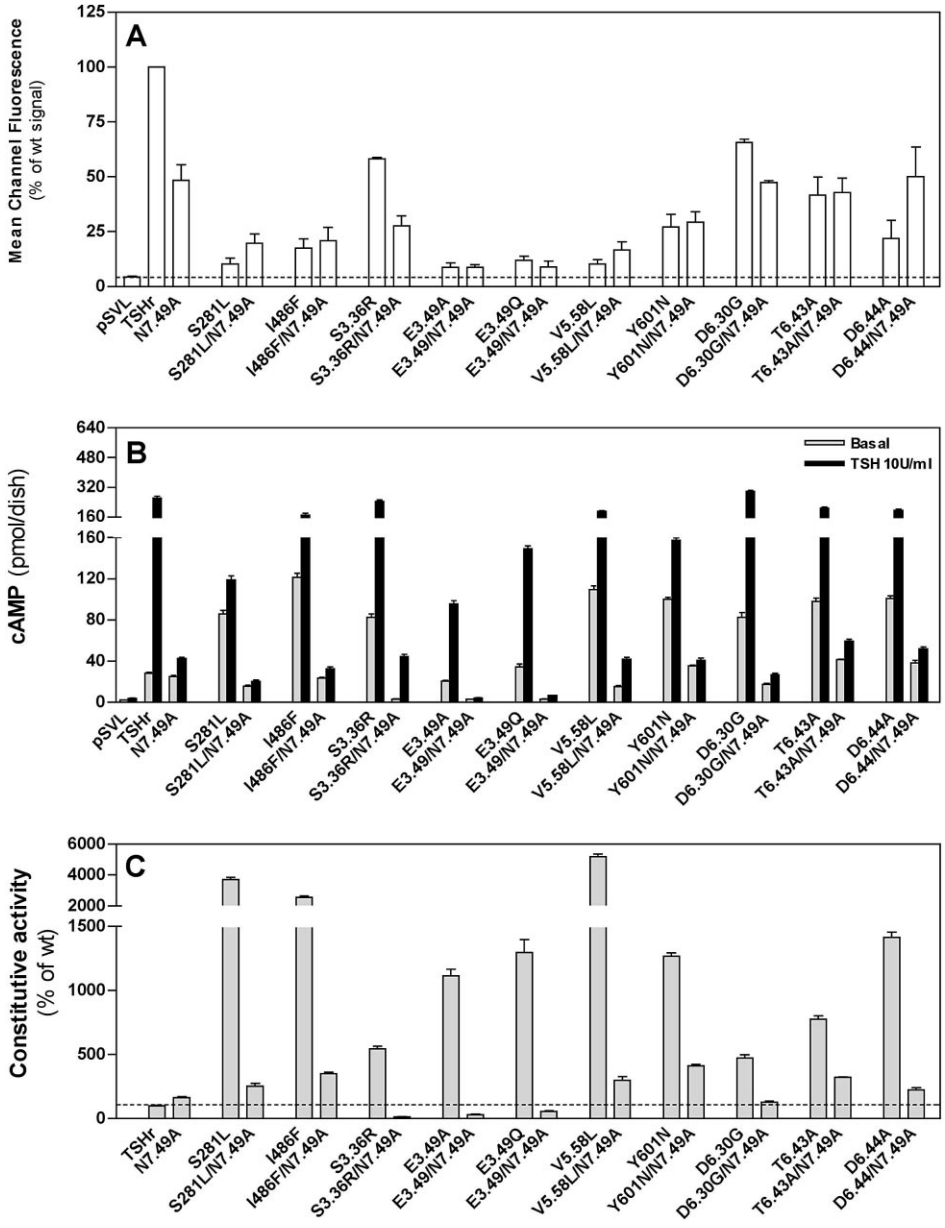

Fig. 2. N*7.49*A substitution reduces the basal activity of activating TSHr mutants. A: Level of expression of the receptors in transfected COS cells. Cell-surface expression of wild-type TSHr and the different mutants was measured by FACS using the BA8 monoclonal antibody, which recognizes a conformational epitope in the N-terminal domain of the receptor. Values represent mean cell fluorescence, normalized to the value obtained for wild-type TSHr. B: Basal and TSH-stimulated cAMP accumulation. cAMP accumulation was measured in COS cells transfected with each receptor construct as described in Section 2. TSH was added at 10 mU/ml during 1 h of incubation. C: Normalized constitutive activity of wild-type and mutant receptors. Basal cAMP accumulation was normalized to the cell-surface expression (see Section 2). This figure presents the data of one typical experiment out of at least three, performed independently (error bars: S.E.M.).

different activating mutations. Would all the mutants be similarly affected by suppression of N*7.49* side-chain, or would some keep their constitutive activity, thus introducing a hierarchy in the activating interactions in the mutants? We transfected all single and double mutants transiently in COS-7 cells and measured surface expression of the receptors by FACS analysis (Fig. 2A). Basal and TSH-stimulated intracellular accumulations of cAMP (Fig. 2B) were quantified and the constitutive activity of the individual constructs was estimated by normalizing basal cAMP values to receptor expression (see Section 2 and [13]). The normalized basal activity of the various single mutants ranged between 570% and 5192% of the constitutive activity of the wild-type TSHr. Addition of the N*7.49*A amino acid substitution to individual mutants decreased dramatically their constitutive activity in all cases (Fig. 2C). For example, normalized basal activity of S281L was reduced from 3695% to 253% of wild-type, activity of V*5.58*L was reduced from 5192% to 298% and activity of D*6.30*G was reduced from 470% to 127%. Thus, none of the receptor constructs was immune to inhibition of its constitutive activity by the N*7.49*A amino acid substitution, whether harboring a mutation close to the site of interaction with TSH in the ECD (S281L) or in the region putatively involved in interaction with the G protein (E*3.49*, D*6.30*G).

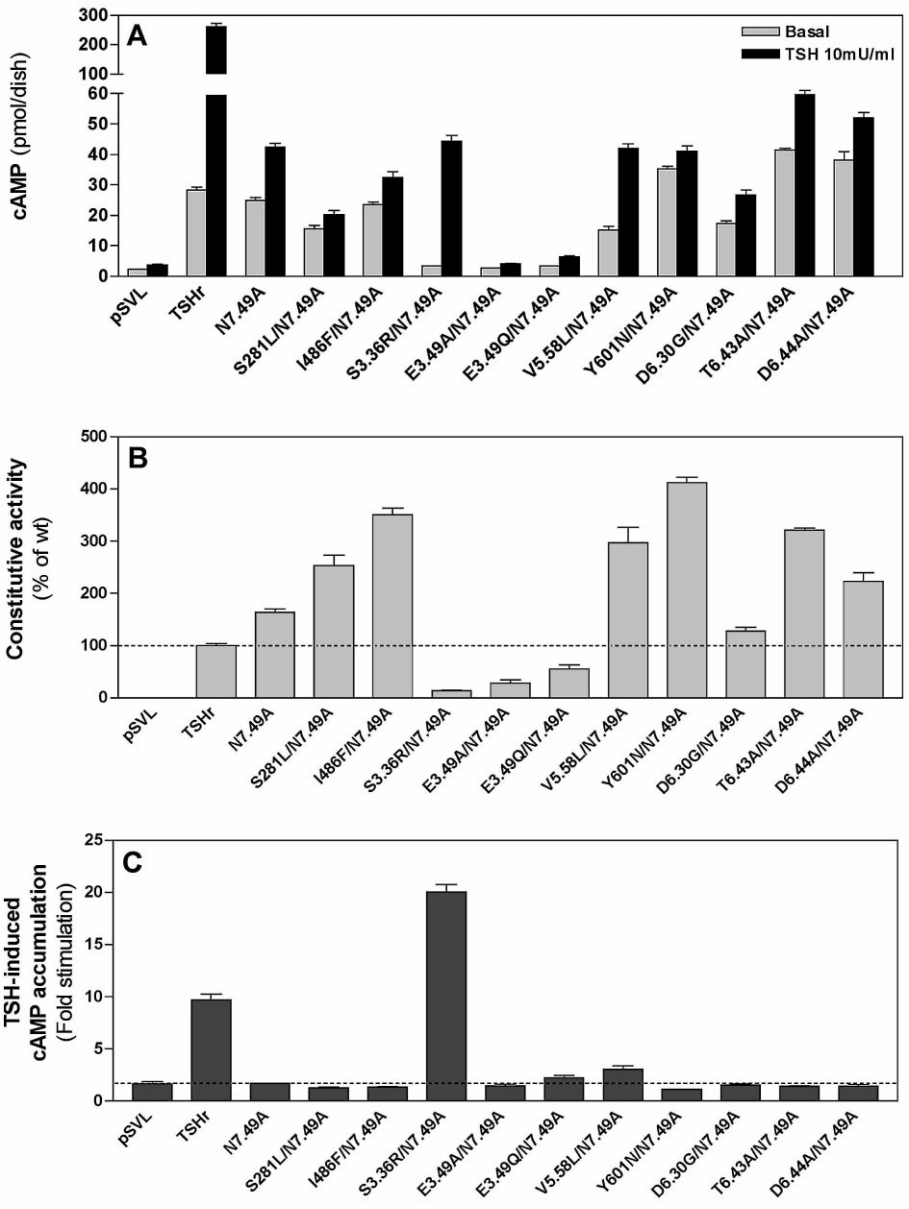

Fig. 3. Comparative effect of N*7.49*A on the different TSHr activating mutants. A: Basal and TSH-stimulated cAMP accumulation in transfected COS cells. cAMP accumulation was measured for each receptor as described in Section 2. TSH was added at 10 mU/ml during 1 h of incubation. B: Normalized constitutive activity of wild-type and mutant receptors. Basal cAMP accumulation was normalized to the cell-surface expression (see Section 2). C: TSH-induced cAMP production. The results are expressed in fold stimulation above basal for each mutant (see Section 2). This figure presents the data of one typical experiment out of at least three, performed independently (error bars: S.E.M.).

Our results indicate that N*7.49* must be involved in a network of interactions shared by the active conformation(s) achieved in all individual mutants as well as in the wild-type receptor stimulated by TSH. Of interest, the N*7.49* mutation affects equally the constitutive activity of mutants with a different ability to couple to Gs and Gq (e.g. I486F and D*6.30*G [23]). The importance of N*7.49* in the activation of rhodopsin-like GPCRs by their natural agonists has been documented in many receptor species [24–26]. The present results indicate that the molecular interaction(s) of this residue are crucial to stabilizing the active conformation(s) of the TSHr and cannot be bypassed, even by activating mutations affecting residues very close to the site of interaction with the G proteins.

3.2. Some N7.49A double mutants lose virtually all basal activity

As already noted in Govaerts et al. [13], the N*7.49*A single mutant, while losing the ability to be stimulated by TSH, kept a normalized basal activity comparable to the wild-type receptor (Fig. 2C). Similarly, in seven of the ten mutants studied, suppression of the side-chain of N*7.49* decreased strongly their normalized constitutive activity, but never to a level below that of the wild-type TSHr (Fig. 3A,B). Three mutants in TM3 make exception, E*3.49*A, E*3.49*Q and S*3.36*R, with a normalized constitutive activity of the double mutants significantly lower than that of the wild-type TSHr. In particular, the S*3.36*R/N*7.49*A double mutant which was relatively well expressed at the cell surface (27.8 ± 4.3% of the

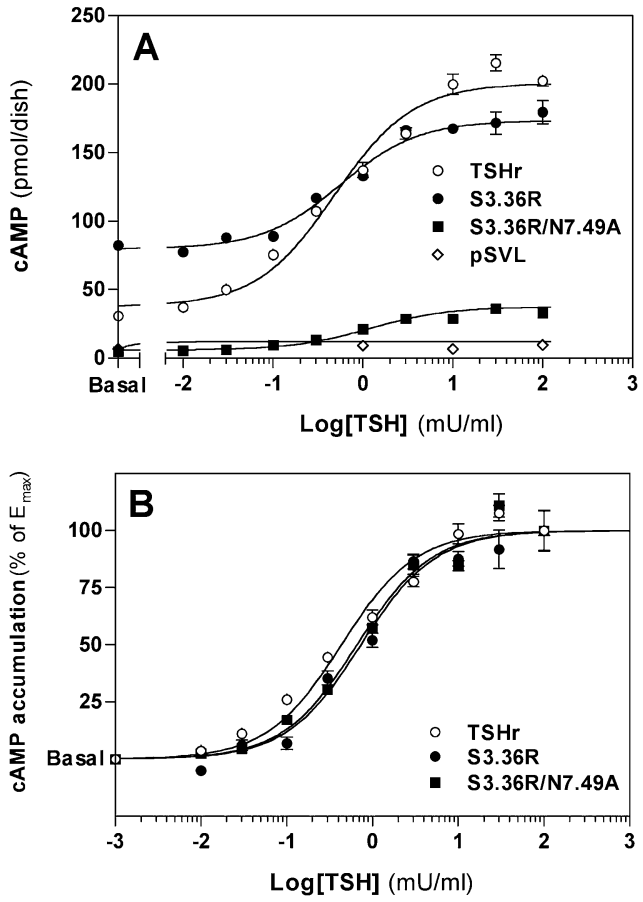

Fig. 4. TSH stimulation of the S*3.36*R/N*7.49*A mutant. TSH-induced cAMP accumulation in COS cells transfected with the different mutants. The curves represent a typical experiment out of at least three, performed independently. Results were analyzed by non-linear regression using the GraphPad Prism software. A: Absolute cAMP values are shown in this panel, demonstrating the ability of TSH to induce significant response in the mutant S*3.36*R/N*7.49*A. B: Data were normalized between basal and maximum values (E_{max}).

wild-type), displayed a basal activity close to zero (compare the cAMP values achieved in pSVL-transfected cells, 2.28 ± 0.06 pmol/dish, and in S*3.36*R/N*7.49*A transfected cells, 3.30 ± 0.06 pmol/dish; Fig. 3). The differential effect of the N*7.49*A mutation on the basal activity of the wild-type TSHr (no effect) and its ability to be stimulated by TSH (strong inhibition) [13], and the present observation that some double mutants are silenced below the level of the wild-type TSHr, while other keep wild-type-like basal activity, have profound implication for our understanding of the activation mechanisms. It suggests that basal and agonist- or mutation-induced activation may be stabilized by different molecular interactions. Strikingly, a molecular explanation must be found for the observation that, when added on a wild-type background, the S*3.36*R or E*3.49*A/Q substitutions cause increased constitutive activity whereas, when the same mutations are engineered on an N*7.49*A background (*presenting the same basal activity as the wild-type TSHr*), the result is partial or total silencing. The involvement of E/D*3.49* in the activation of rhodopsin-like GPCRs has been widely recognized [20–22,27,28] and residues in the middle of the TM3 helix have been implicated in the recognition of agonists [29–31]. With the possibility that some of the activating mutations of the TSHr in TM3 (e.g. S*3.36*R) might trigger local conformational changes similar to those resulting from the binding of agonists in receptors to small ligands, the present observations may lead to a molecular dissection of these changes.

3.3. N7.49 is a key residue in the activation of the TSHr

In agreement with observations made previously with the N*7.49*A single mutant, the double mutants lose their ability to respond to TSH (Fig. 3C). A particular case is S*3.36*R/N*7.49*A since at 10 mU/ml of TSH this double mutant displayed more than 20-fold stimulation of its basal cAMP level, when all other double mutants remained virtually unaffected (Fig. 3).

Concentration-effect curves (Fig. 4A,B) showed that S*3.36*R/N*7.49*A presents a similar EC_{50} as the wild-type TSHr or the S*3.36* single mutant (0.67 ± 0.32; 0.44 ± 0.10 and 0.74 ± 0.19, respectively). However, the *maximal* activity it reaches is low when compared to that of the wild-type TSHr, being similar to the *basal* activity of most of the other double mutants and the wild-type TSHr. For comparison, and as expected, the N*7.49*A single mutant was almost insensitive to TSH (Fig. 3C).

The reason for the exceptional behavior of the S*3.36*R/N*7.49*A double mutant is not clear. One may argue that it keeps responding to TSH because of its exceptionally low basal activity. This, in turn, would suggest that the other N*7.49*A mutants would actually be kept in a 'maximally (though modestly efficient) stimulated state'. According to this view, it would be the peculiar conformation of S*3.36*R/N*7.49*A (see discussion above) which would be responsible both for its low basal activity and for allowing it to respond to the structural changes induced by the agonist. However, a low basal activity is not enough to allow for stimulation of the double mutants by TSH; the E*3.49*A/N*7.49*A and E*3.49*Q/N*7.49*A double mutants, which display a normalized basal activity much lower than the wild-type TSHr, have lost the ability to be stimulated by TSH (Fig. 3).

4. Conclusion

Our experiments identify N*7.49* as a key residue in the activation mechanism of the TSH receptor by its natural agonist and, more importantly, by a series of very diverse activating mutations, including those affecting the canonical E/DRY motif. They also demonstrate that basal activity and agonist- or mutation-stimulated activity might involve different structural changes. The challenge is now to identify the partners of N*7.49* as well as the mosaic of interactions stabilizing the active state(s) of the TSHr. The availability of a particularly large panel of activating mutations should make this a realistic endeavor for the TSHr by a combination of molecular modeling, functional assays and site-directed mutagenesis.

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