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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 N7.49 (Asn⁶⁷⁴) in TM7 is mandatory for activation of the TSHr, 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 N7.49 (N7.49A double mutants). In addition, comparative effects of the N7.49A substitution on the ten mutants demonstrate that basal activity and agonist- or mutation-stimulated activity might involve different structural changes.

Keywords:

GPCR; TSH receptor; constitutive activity; natural mutations; activation mechanism

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Abbreviations:

TSH, Thyroid-Stimulating Hormone; GPCR, G Protein Coupled Receptor; TM, transmembrane helix; FACS, Fluorescence Activating Cell Sorting

1. Introduction

Glycoprotein hormones receptors (TSHr, LH/CGr, 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 7 α -helices (7 TM), responsible for signal transduction. Binding of the hormones to the ECD triggers conformational changes of the 7 TM 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 aminoacid 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 N7.49⁶⁷⁴ (see Methods for description of the numbering system) was found to interact with a conserved Asp in TM6 (D6.44⁶³³). Breaking of this interaction (e.g. as a consequence of natural or artificial mutations affecting D6.44⁶³³) was proposed to release N7.49, 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 N7.49A 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 N7.49 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 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 N7.49 is the asparagine in transmembrane helix 7 (TM7), located 1 residue before the highly conserved proline P7.50. Residue D2.50 corresponds to Asp 460 in the TSHr numbering; S3.36 to Ser 505; E3.49 to Glu 518; V5.58 to Val 597; T6.43 to Thr 632; D6.44 to Asp 633 and N7.49 to Asn 674. For residues located outside the transmembrane helices (S281 in the ECD and I486 in the first extracellular loop, Y601 and D6.30 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 (DMEM) supplemented with Fetal Bovine Serum 10%, sodium pyruvate 1 mM, penicillin 100 UI/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 fluoresceine-conjugated gamma chain-specific goat anti-mouse IgG (SIGMA, chemical CO, St Louis, MO). 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 minutes, 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, Chemical CO, St Louis). 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 FACS value (= fluorescence of receptor-transfected cells – fluorescence of pSVL-transfected cells), which can be summarize 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 wt 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))]).

3. Results and Discussion

3.1. A common "conformational pathway" in all activating mutation 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 aminoacid 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 (S3.36⁵⁰⁵R, V5.58⁵⁹⁷L, T6.43⁶³²A, D6.44⁶³³A), or at the border between TMs and intracellular loops (Y601N, D6.30G). 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 (R3.50 of the E/DRY motif). Information available from other GPCRs predicts that the E3.49A and E3.49Q 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 D6.44, a conserved residue in TM6 of the glycoprotein hormone receptors. Disruption of this interaction, by mutation of D6.44 or secondary to stimulation by TSH, was proposed to release the side-chain of N7.49, 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 N7.49A substitution when it is added on the background of each of the ten different activating mutations. Would all the mutants be similarly affected by suppression of N7.49 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 accumulation 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 Materials and Methods 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 N7.49A aminoacid 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 wt, activity of V5.58L was reduced from 5192 % to 298 % and activity of D6.30G was reduced from 470 % to 127 %. Thus, none of the receptor constructs was immune to inhibition of its constitutive activity by the N7.49A aminoacid 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 (E3.49, D6.30G). Our results indicate that N7.49 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 N7.49 mutation affects equally the constitutive activity of mutants with a different ability to couple to Gs and Gq (e.g. I486F and D6.30G, [23]). The importance of N7.49 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 by-passed, even by activating mutations affecting residues very close to the site of interaction with the G proteins.

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

As already noted in Govaerts et al [13], the N7.49A 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 N7.49 decreased strongly their normalized constitutive activity, but never to a level below that of the wt TSHr (Fig. 3A, B). Three mutants in TM3 make exception; E3.49A, E3.49Q and S3.36R, with a normalized constitutive activity of the double mutants significantly lower than that of the wt TSHr. In particular, the S3.36R/N7.49A double mutant which was relatively well expressed at the cell surface ($27,8 \pm 4,3$ % of the wt), displayed a basal activity close to zero (compare the cAMP values achieved in pSVL-transfected cells, 2.28 ± 0.06 pmol/dish, and in S3.36R/N7.49A transfected cells, 3.30 ± 0.06 pmol/dish; fig. 3). The differential effect of the N7.49A mutation on the basal activity of the wt 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 wt TSHr, while other keep wt-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 to the observation that, when added on a wt background, the S3.36R or E3.49A/Q substitutions cause increase constitutive activity whereas, when the same mutations are engineered on a N7.49A background (*presenting the same basal activity as the wt TSHr*), the result is partial or total silencing. The involvement of E/D3.49 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. S3.36R) 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 N7.49A single mutant, the double mutants lose their ability to respond to TSH (Fig. 3C). A particular case is S3.36R/N7.49A since at 10 mU/ml of TSH this double mutant displayed more than twenty-fold stimulation of its basal cAMP level, when all other double mutants remain virtually unaffected (Fig. 3).

Concentration-effect curves (Fig. 4A, B) showed that S3.36R/N7.49A presents a similar EC_{50} as the wt TSHr or the S3.36 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 wt TSHr, being similar to the *basal* activity of most of the other double mutants and the wt TSHr. For comparison, and as expected, the N7.49A single mutant was almost insensitive to TSH (figure 3B)

The reason for the exceptional behavior of the S3.36R/N7.49A 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 N7.49A mutants would actually be kept in a "maximally (though modestly efficient) stimulated state". According to this view, it would be the peculiar conformation of S3.36R/N7.49A (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 E3.49A/N7.49A and E3.49Q/N7.49A double mutants, which display a normalized basal activity much lower than the wt TSHr, have lost the ability to be stimulated by TSH (Fig. 3).

Conclusion

Our experiments identify N7.49 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 N7.49 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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Figure legends

Figure 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 Materials and Methods for description of the numbering system.

Figure 2. The N7.49A substitution reduces the basal activity of activating TSHr mutants.

A. Level of expression of the receptors in transfected COS cells. Cell surface expression of wt 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 wt TSHr. B. Basal and TSH-stimulated cAMP accumulation. cAMP accumulation was measured in COS cells transfected with each receptor construct as described in Materials and Methods. TSH was added at 10mU/ml during one hour of incubation. C. Normalized constitutive activity of wt and mutant receptors. Basal cAMP accumulation was normalized to the cell-surface expression (see Materials and Methods).

This figure presents the data of one typical experiment out of at least three, performed independently (error bars: s.e.m).

Figure 3. Comparative effect of N7.49A 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 Materials and Methods. TSH was added at 10mU/ml during one hour of incubation. B. Normalized constitutive activity of wt and mutant receptors.

Basal cAMP accumulation was normalized to the cell-surface expression (see Materials and Methods). C. TSH-induced cAMP production. The results are expressed in fold stimulation above basal for each mutant (see Materials and Methods). This figure presents the data of one typical experiment out of at least three, performed independently (error bars: s.e.m.).

Figure 4. TSH stimulation of the S3.36R/N7.49A 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 nonlinear 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 S3.36R/N7.49A. B. Data were normalized between basal and maximum values (E_{\max}).

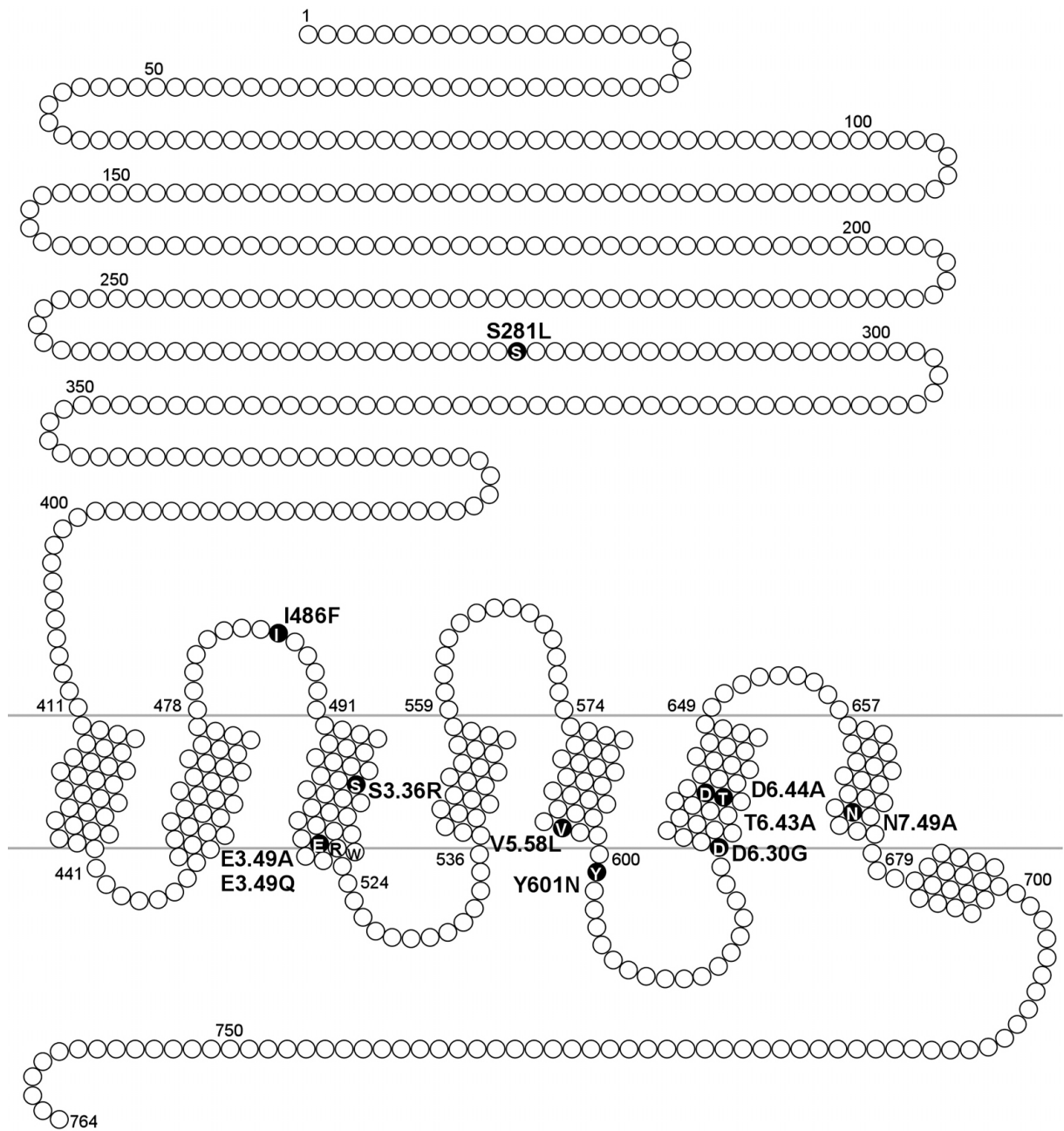


Figure 1

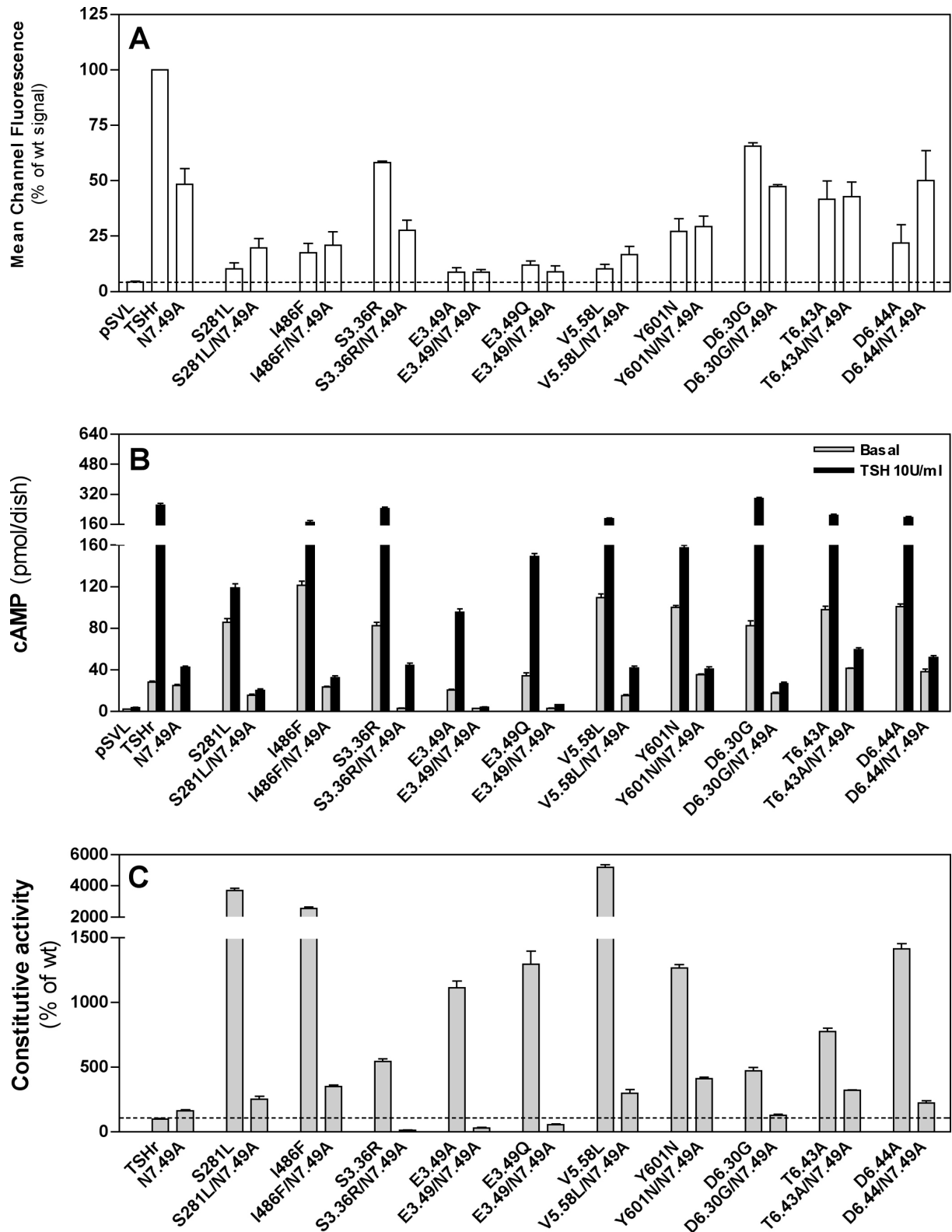


Figure 2

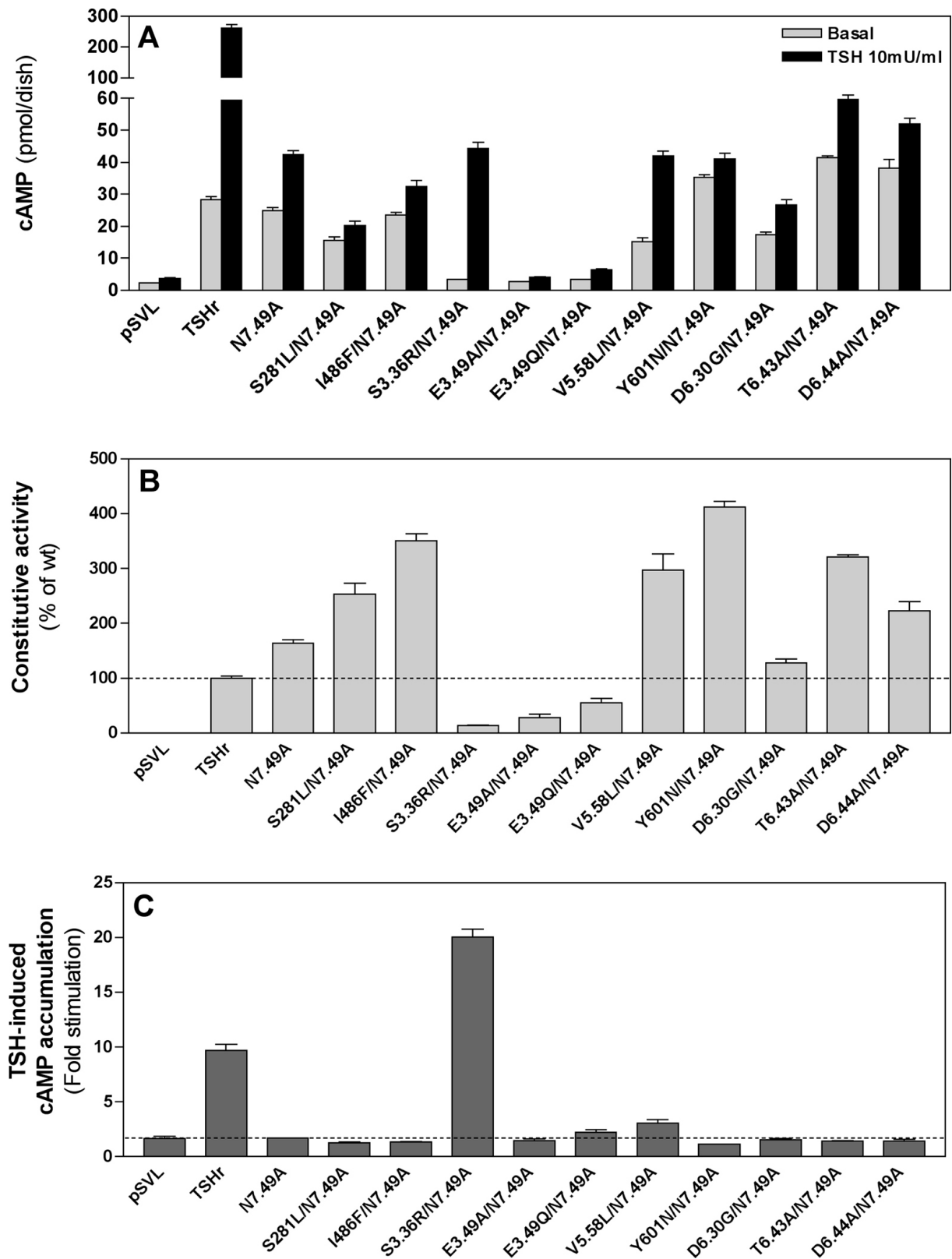


Figure 3

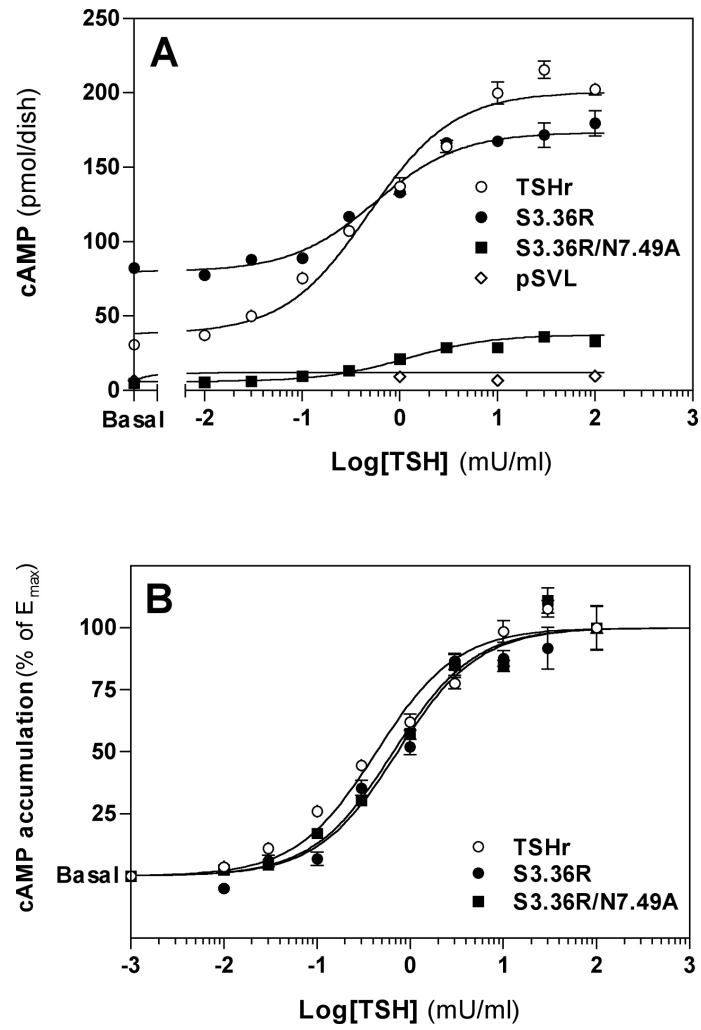


Figure 4