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Characterization of the molecular motions of constitutively active G protein-coupled receptors for parathyroid hormone

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Abstract

The molecular mechanism of constitutive activity of the G protein-coupled receptor for human parathyroid hormone (PTH1) has been examined by molecular dynamics (MD) simulations. The single point mutations H223R, T410P, and I458R, of the PTH1 receptor result in ligand-independent receptor activation. Extensive MD simulations indicate that each of the mutations, through different mechanisms, lead to very similar conformational changes of the third intracellular loop. The structural changes, centered on K405 in the C-terminus of the third intracellular loop, can be traced back to the single-point mutations by calculation of the forces and torques responsible for the collective motions of the receptor. This analysis indicates a direct correlation between the conformational preferences of the cytoplasmic loop and the mutations in different locations of the receptor: TM2 (H223R), TM6 (T410P), and TM7 (I458R). Given the pivotal role of the third intracellular loop of PTH1 in coupling to the G proteins, the structural changes induced by these single-point mutations may be responsible for the ligand-free activation of the receptor. These results coupled with the high-resolution structure of the third cytoplasmic loop of PTH1, previously determined in our laboratory, provide unique insight into the mechanism of ligand free activation of the PTH1 receptor.

Keywords: Parathyroid hormone; PTH receptor; G protein-coupled receptors; Coupling of G proteins to PTH receptor; Constitutively active receptors; Structure intracellular loop 3

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1. Introduction

Parathyroid hormone (PTH) plays a pivotal role in the regulation of extracellular calcium homeostasis [1,2]. PTH, along with vitamin D, is responsible for maintenance of serum calcium levels required for cellular function, adjusting for the variations from bone remodeling, renal function, and dietary intake of calcium. These physiological actions are mediated through the activation of the PTH receptor, PTH1, a member of the G protein-coupled receptor (GPCR) super family of heptahelical, transmembrane signaling proteins [3,4].

In 1996, Jüppner and co-workers [5,6] reported two single point mutations, H223R and T410P, in PTH1 which led to constitutively active receptors, ligand independent activity enhanced compared to basal levels. These mutations are related to Jansen's Metaphyseal Chondrodysplasia, a rare disease associated with hypercalcemia [5–7]. More recently, a third single point mutation, I458R, was observed in a patient with Jansen's disease [7,8]. It has been shown that in mice the presence of the constitutively active receptor can overcome the effects of absence of PTH [9]. In addition to their medicinal relevance and usefulness as biochemical tools, the constitutively active receptors provide unique information into the molecular mechanism of receptor activation.

Here, we characterize the consequences of the three naturally occurring single point mutations (H223R, T410P, I458R) in the human PTH1 receptor using extensive molecular dynamics (MD) simulations. The simulations were carried out using a novel membrane mimetic which allows for extensive calculations while maintaining the overall, biphasic character of the membrane environment [10,11]. From analysis of the simulations following established procedures [12], we correlate the single point mutations to short and long range effects on the receptor structure. Despite being dispersed through out the receptor in transmembrane helices (TM) 2, 6, and 7, (see Fig. 1), all of these substitutions lead to similar changes in the third cytoplasmic loop (IC3), centered on K405. Coupling these results with the high-resolution structure of IC3 of PTH1 [13,14], provides insight into the molecular mechanism of ligand-independent receptor activation.

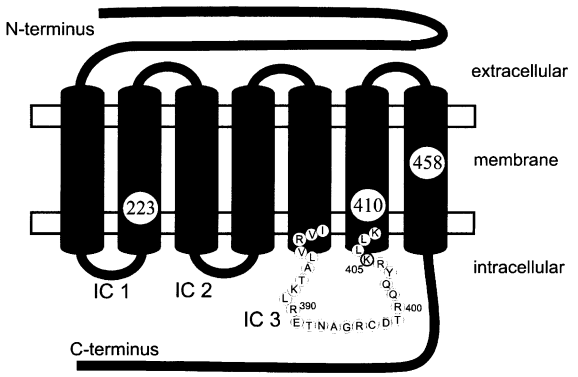

Fig. 1. Schematic diagram of the human PTH1 receptor. The positions of the three sites of substitution in the mutant receptors are indicated. The sequence of the third cytoplasmic loop, IC3, is given.

2. Methods

2.1. Model building

Modeling of the transmembrane (TM) domain of the human PTH receptor (accession number: Q03431) was carried out with the WHATIF program [15] as described previously [11,16]. The method utilizes rhodopsin and bacteriorhodopsin [17,18] as templates for the topological orientation and arrangement of the seven membrane-spanning helices. The receptor model was completed by addition of loops, generated using a metric matrix based DG program employing distances between adjacent TM helices as restraints.

The model included the structure and topological orientation of the loops and termini of PTH1 which have been examined by NMR in a lipid environment, including the fragments PTH1 (168–198) and PTH1(330–365), which consist of the proximal N-terminus and the third intracellular loop of PTH1 [10,14]. As this work focuses on structure and dynamics of the TM domain and IC loops, N-terminal residues before T178 as well as C-terminal residues after S475 were not included in the simulations.

Following previously published procedures [16], the BLAST program [19] was used to search for homologous regions between the loops and termini of the receptor and proteins of known three-dimensional structure within the Brookhaven Protein Data Bank (PDB). Each homologous PDB hit was then analyzed for secondary structure. In cases where multiple occurrences of similar secondary structure were observed for a given region of the receptor, this secondary structure was incorporated into the model. Taking into account the difficulty in determining the partner strand in the formation of a β-sheet, the homology analysis was restricted to α-helices.

The resulting wild type receptor model was energy minimized and subjected to a short MD run using the DISCOVER program (Molecular Simulations Inc.) in order to remove initial strains. During this stage the backbone topology of the TM helices was preserved by constraining all backbone heavy atoms to their initial positions. Similarly, regular helical secondary structure (as determined above) of loops and termini was maintained using dihedral angle restraints.

Starting structures of the receptor mutants were obtained by replacing the respective wild type residues by those found in mutated receptors. At the same time the side-chain conformation of the native residue was transferred to its mutant counterpart. In order to remove clashes, the resulting structure was minimized with the TM backbone topology fixed as described above.

2.2. MD simulations

The receptor models were placed in a three-layer (water/decane/water) simulation cell for further refinement by MD simulations in a membrane-mimicking environment. The TM helices were placed in the decane layer with the extra- and intracellular regions embedded in the water phases. The lipid bilayer was mimicked by a layer of approximately 40 Å of decane molecules, with approximately 30-Å layers of water below and above [20]. For sake of computational simplicity, the solvent system neglects the charged nature of the lipid/water interface while maintaining the overall biphasic, hydrophobic/hydrophilic character as well as the molecular motions of the long acyl-chains found in membranes [20].

The refinement consisted of three stages. During the first 50 ps of MD the solvent was equilibrated while the receptor was restrained. Next, the loops and termini were released and allowed to equilibrate in the course of another 100 ps. The resulting system configuration served as a starting point for two 200-ps runs set up with different initial velocities. At this stage helices were maintained by appropriate backbone distance restraints. Taking into account the special structural and dynamic properties introduced by Pro kinks in α-helices, portions of TM helices before and after a Pro residue were allowed to evolve independently. A representative structure of each receptor was obtained by energy minimization of the average structure over the last 100 ps of the trajectories. To probe the stability of the wild type receptor model, one of the final simulations of the wild type receptor was extended to 1 ns. All simulations were carried out using periodic boundary conditions. Neighbor lists were updated every 10 steps. Only non-bonded interactions within 8 Å (Lennard-Jones) and 10 Å (Coulomb) were included, respectively. The simulation temperature of 300 K was maintained via temperature bath coupling with a time constant of 0.02 ps [21]. All simulations were performed on an SGI Origin 2000 computer using the GROMACS package [22].

2.3. Analysis of domain motions

Concerted movements of groups of atoms interacting with each other are not uncommon and have been shown to be essential for protein function [12,23–26]. In the trajectories obtained from the MD simulations of our receptor models, the seven TM helices act as separate domains in which the component atoms exhibit collective motions. These collective motions of atoms can be considered to represent a rigid domain motion.

The analysis of motions of the TM helices was carried out following the procedure described by

Weinstein and co-workers [12]. Utilizing classical mechanics, the method describes the motion of a domain by two physical quantities: (1) the domain's momentum and angular momentum, which describe how the domain translates and rotates, respectively (e.g. upon introduction of a mutation); and (2) the force and torque exerted on a domain, which describe how inter-domain interactions influence the motion of the domain. Briefly, the direction of the overall translation and rotation of the TM helix in the mutant with respect to the wild type are calculated as unit vectors, $\mathbf{n}_P^D$ and $\mathbf{n}_J^D$, respectively.

$$\mathbf{n}_P^D = \left[\sum_{i \in D} m_i(\mathbf{r}_i^M - \mathbf{r}_i^W)\right] / |\sum_{i \in D} m_i(\mathbf{r}_i^M - \mathbf{r}_i^W)| \quad (1)$$

$$\mathbf{n}_J^D = \left[\sum_{i \in D} m_i \mathbf{r}_i^W \times (\mathbf{r}_i^M - \mathbf{r}_i^W)\right] / |\sum_{i \in D} m_i \mathbf{r}_i^W \times (\mathbf{r}_i^M - \mathbf{r}_i^W)| \quad (2)$$

where m_i is the mass of atom i, $\mathbf{r}_i^W$ is the position vector of atom i in the wild type and $\mathbf{r}_i^M$ is the corresponding vector in the receptor mutant. $\Sigma_{i \in D}$ implies summing up over all the atoms in helical domain defined as D. The effect of another domain, defined as B, on the translation and rotation of D at time t during the MD simulation can be characterized by the force $\mathbf{F}_B^D(t)$ and torque $\mathbf{T}_B^D(t)$ exerted on D by B. The energetic contributions from the helical interactions can be calculated using the GROMACs parameter set used for the MD simulation. Forces resulting in projections larger than zero support the overall translation of domain D (force has a component parallel to the overall translation), whereas negative projection values oppose the overall translation (force has a component antiparallel to the overall translation). Analogous considerations apply for the rotation of the domain. This procedure allows for the analysis of the specific interactions, forces and torques, which lead to the observed translation or rotation. The structural consequences of the receptor mutations, even if involving many sequential interactions, can be unambiguously identified.

3. Results

For a number of GPCRs, the extracellular N-terminus of the receptor is not required for the constitutive activity [27–29]. Our analysis of the native and mutant receptors of PTH1 is therefore focused on the relative motions of the TM helices and characterizing the effects these motions have on the intracellular domains. Utilizing procedures outlined by Weinstein and co-workers [12], the collective motions of the TM helices in the wild type and mutant receptors are compared and the forces and torques which produced these motions are back calculated. The findings reported below are the result of two complete MD simulations carried out for the native and each mutant receptor.

3.1. Wild type PTH1 receptor

The simulations of the wild type receptor display no major displacements (RMSD less than 1.1 Å) of the TM helices. This is an indication that the receptor within the membrane mimetic is energetically stable. Using the concept of protein microdomains, the results of each of the mutant receptors are compared to the native receptor.

3.2. The I458R mutation

Residue 458 is located approximately in the middle of TM7, slightly closer to the IC end of the helix. Substitution of an isoleucine by arginine in the interior of the bundle leads to a number of steric conflicts, which are resolved by topological rearrangements within the 7-helical bundle. This rearrangement is most pronounced for TM helices 1, 2 and 6 (an RMSD of 1.6 Å); only minor adjustments of the relative orientations of TM helices 3, 4 and 5 are observed (RMSD of 0.8 Å). For the comparison of the results of the I458R mutant and wild type receptors, the heavy backbone atoms of TM4 were superimposed.

During the simulation of I458R, TM7 translates and tilts towards TM6 (RMSD 5.1 Å with respect to TM7 in the wild type) with the EC and IC termini of TM6 5.8 and 3.1 Å, respectively,

away from their positions in the wild type receptor. The substitution also affects the topological orientation of TM6. Because of the proline, P415, in the center of TM6, the helix is treated as two separate domains: an extracellular half, TM6e, consisting of residues 416–427, and an intracellular half, TM6i, consisting of residues 406–414. During the simulation, TM6 moves away from the center of the bundle (backbone RMSDs of 2.7 and 1.7 Å, with the native receptor for TM6e and TM6i, respectively). It is well established that prolines lead to increased flexibility of helices [30]. In our model of the I458R mutant, the kink around P415 between TM6i and TM6e increases from 20.7 to 23.6°.

A more quantitative analysis of these observations is provided by the calculation of the forces and torques imparted on TM6 by TM7, shown in Fig. 2. In Fig. 2a, the positive torque indicates that TM7 is supporting the rotation of TM6e. The plot of the force indicates an original interaction between TM7 and TM6e, which opposes the translation of TM6 (indicated by a negative value), followed by a positive interaction, reinforcing the translation, between the two TM helices. Similar conclusions are drawn for the interaction between TM6i and TM7 (shown in Fig. 2b): TM7 imparts a torque to TM6i supporting the rotation, while the negative force indicates that the substitution is opposing the translation of TM6i. These interactions approach zero during the end of the simulation.

The interactions between TM6 and TM7 cause IC3 to shift towards TM5, as illustrated in Fig. 3. The accessible conformational space of IC3 is restricted by its high helical content, as experimentally determined by NMR [13,14]. The magnitude of the observed conformational change in the C-terminal region of IC3 is greatest for K405, situated close to TM6. In wild type, K405 is directed towards the center of the bundle and the N-terminal region of IC2 while in the I458R mutant, K405 projects towards TM5 the center of IC3 (Fig. 3). The displacement of the side chain of K405 compared to wild type is 9.5 Å (using the $^{\varepsilon}$N), and it approaches the formation of a salt bridge with E391 (5.5 Å between the charged side chains).

3.3. The H223R mutation

Similar to the I458R mutant, significant rearrangements are observed for TM1, TM2 and TM6 (backbone RMSD 1.5 Å) during the simulations of the H223R mutant receptor. Residue 223 is located at the IC end of TM2 and upon introduction of the mutation, there is a tilting of TM2 (backbone RMSD 2.6 Å with respect to the wild type): the EC end of TM2 tilts towards TM3, while the IC portion stays in close proximity to

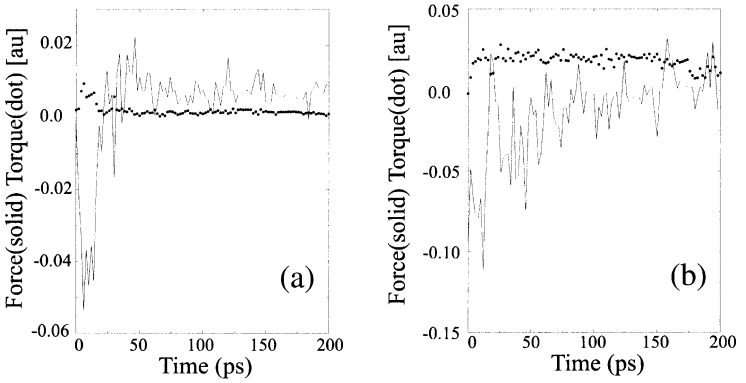

Fig. 2. The time dependence of the force (solid line) and torque (dots) (given in a.u.) observed for the I458R mutant receptor: (a) exerted on TM6e (the e denotes the extracellular portion of TM6) by TM7; and (b) exerted on TM6I by TM7. The values are projected on the direction of the translation or rotation of the helices observed during the simulation.

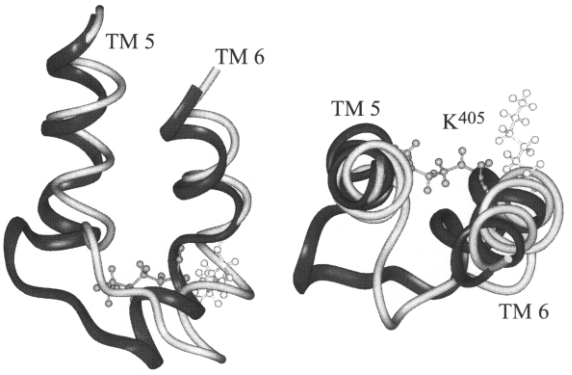

Fig. 3. Superposition of structures from the simulations of the wild type (gray) and the I458R mutant (dark) PTH1 receptor. The heavy backbone atoms of TM4 were used for the superposition. The side chain atoms of K405 are illustrated as ball-and-stick.

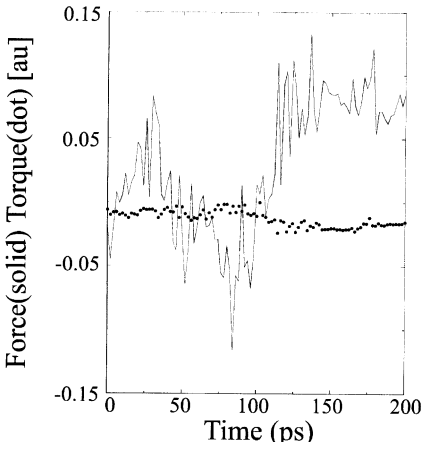

Fig. 4. The time dependence of the force (solid line) and torque (dots) from TM2 applied to TM7 calculated from the MD simulation of the H223R mutant receptor.

TM7. This enables R223 to form a salt bridge with E465 (closest interresidual atom–atom distance: 3.7 Å). The formation of a salt bridge between R223 (TM2) and E465 (TM7) is also supported by translation of TM7 towards TM2, accompanied by a slight rotation which brings both residues closer together. Analysis of the forces between TM2 and TM7 indicate (Fig. 4) that the origin of the translation of TM7 emanates from TM2.

The perturbation further propagates from TM7 to TM6, causing TM6i to move towards the center of the bundle (backbone RMSD 2.3 Å with respect to wild type) and undergo a slight rotation. TM6e is altered much less (backbone RMSD 1.1 Å with respect to the wild type), which leads to an increase in the helical kink of P415 to 35.5°. This stepwise perturbation is corroborated by the back calculation of strong interactions (force and torque) between TMs 2, 7 and 6 (data not shown). Although spatially close to TM6, the conformation and topological orientation of TM5 remain unchanged (backbone RMSD 0.8 Å with respect to the wild type).

As in the I458R mutant, the motion of TM6 causes a change in the conformation of IC3, bringing about a reorientation of K405, which now projects towards TM5 and the center of the IC3 loop (Fig. 5). The magnitude of this reorientation is clearly illustrated by the distance, 7.9 Å, between $^{\varepsilon}$N of K405 in the mutant and wild type receptors. In addition the N-terminal residues of IC3 undergo no significant motion (backbone RMSD 1.0 Å for residues 384–388 with respect to wild type).

3.4. The T410P mutation

The T410P mutation is located one helical turn away from the IC end of TM6 and is expected to

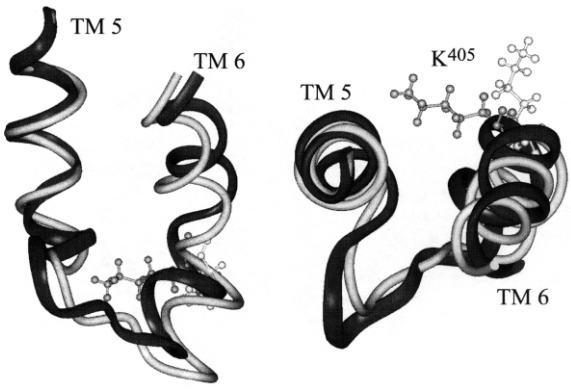

Fig. 5. Superposition of structures from the simulations of the wild type (gray) and the H223R mutant (dark) PTH1 receptor. The heavy backbone atoms of TM4 were used for the superposition. The side chain atoms of K405 are illustrated as ball-and-stick.

introduce a second kink to the helix. Indeed, an angle of 27.8° is measured between TM6i (consisting of residues 406–410) and TM6m (consisting of residues 410–415). This second kink is accompanied by a diminishing of the angle around P415, to a value of 3.0°. During the simulation, the topological arrangement of TM3, TM4 and TM5 is not significantly altered (backbone RMSD 0.6 Å with respect to wild type). Considerable domain motions occur for the opposite half of the receptor particularly TM1, TM6 and TM7 (a backbone RMSD 1.8 Å with respect to the wild type).

Compared to wild type, TM6 moves approximately 2 Å towards the cytosol, perpendicular to the plane of the membrane. This leads to the disruption of the salt bridge between K408 and E465 and exposure of K405, located at the IC end of TM6, to the cytosol. Only a minor rotation occurs at the last IC turn of TM5 (V384–K388). This rotation alters the orientation of IC3 continuing from TM5. The result is that K405 projects towards the central seven-helical bundle, similar to wild type, however, at a much more inclined angle, away from the membrane surface and therefore much more solvent accessible.

4. Discussion

From the first report of a constitutively active GPCR, it was recognized as a unique vehicle to probe the mechanism of receptor activation [31–33]. Many of the sites originally found to produce constitutive activity are located in the cytoplasmic portion of the receptor, especially prevalent in the third cytoplasmic loop [31,32,34–37]. Not surprisingly IC3 has been shown to be strongly associated with coupling to the G protein [37–42]. Mechanistically one can imagine that the structural changes of the IC3 introduced by the mutations could lead directly to association with the G protein and initiation of signal transduction.

More recently mutations in the transmembrane helices have been shown to lead to constitutively active receptors [43–46]. These mutations, most often located in TM2, TM5 or TM6, clearly indicate that interactions between the TM helices, stabilizing either the active or inactive form of the receptor, are important for signal transduction. The stabilization of the relative orientation of various TM helices through Coulombic forces or hydrophobic patches has been implicated in a number of receptor systems [45,47–50]. A view of some of the dynamics of the TM domains of rhodopsin during activation has been obtained using specifically placed probes within the helices [51,52].

Here, we utilize MD simulations to probe the manner in which three different single point mutations in the PTH1 receptor lead to constitutive activity. Multiple simulations were run for the wild type and each mutant receptor to obtain average properties. The motions and dynamics of the TM regions were characterized by comparison of the mutant receptors with the results from the native receptor.

Despite the variety of the location (i.e. mutations on TM2, TM6 and TM7) and nature (i.e. $H \rightarrow R$, $T \rightarrow P$ and $I \rightarrow R$) of the mutations examined here, common structural features are induced by these substitutions. Comparing the mutant receptors to the wild type, the structural features and relative topology of TM5 and the N-terminal portion of IC3 (residues 367–388) are similar. The major difference between mutant and wild type is in TM6 and the C-terminal region of IC3. This modification is expected for T410P, a mutation in TM6. For the other two mutants, the structural change in IC3 is the results of concerted inter-TM helical interactions, as illustrated in Figs. 2 and 4. Despite the differing sources of the conformational changes of TM6, all mutations confer a similar structural feature to the C-terminal region of IC3, particularly with respect to K405. In the mutant receptors, K405 reorients towards TM5, becoming more exposed and filling an uncharged gap observed in the wild type receptor. In Fig. 6, the high-resolution structure of the cytoplasmic loop as determined by NMR is displayed [13,14]. In this structure K405 is found projecting outwards, away from TM5 and TM6, with the hydrophobic residues L406 and L407 on one side, and Q401 and Q402 on the other. The mutations examined here alter these

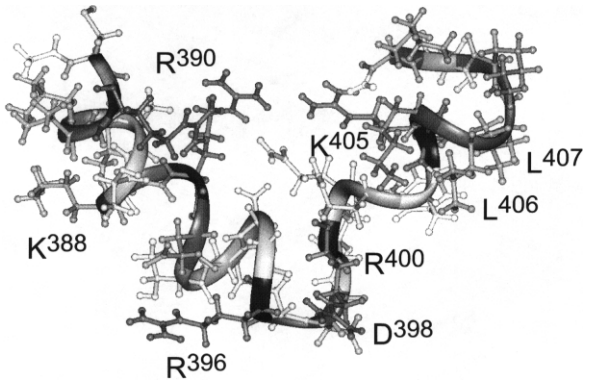

Fig. 6. The structure of the third cytoplasmic loop of the PTH1 receptor as determined by high-resolution NMR [13,14]. Some of the amino acids side chains are labeled.

features, leading to an approximate 90° rotation of the orientation of K405.

The PTH1 receptor has been shown to couple predominately to the G_s-adenylate cyclase and G_q-phospholipase C signaling pathways. The role of the cytoplasmic loops in the coupling to G proteins has been examined by deletions, mutations, and formation of chimera [53,54]. Within IC3, residues V378, L379, T381 and K382 of the opossum PTH1 (corresponding to V384, L385, T387 and K388 of human PTH1) proved to be critical for adenylate cyclase stimulation and/or phospholipase C activation [54]. Replacement of the hydrophobic V384 and L385 with alanine was found to decrease G_q activity, while the T387A mutation altered G_s activity. The charged lysine, K388, residue was found to effect the coupling to both G proteins [54]. Based on the experimentally determined structure of IC3 [13,14], all of these residues of the N-terminal portion of IC3 are part of an α-helix lying on the membrane surface [55], with both T387 and K388 fully exposed. These experimental data clearly indicate that single residues of the cytoplasmic loops can dictate the coupling efficiency of the receptors to the G proteins. From our simulations, we predict that K405 is a major player in the observed ligand independent activation of these mutant receptors. Given the fact that the constitutively active receptors only enhance the cAMP basal levels, we postulate that the C-terminal portion of IC3 is determinant in the coupling to G_s. The concept that the N- and C-termini of IC3 controls the G protein specificity was originally proposed for the muscarinc and adrenergic receptors many years ago [56–58]. Despite the great difference in size of the IC3 domain between PTH1 and these receptors, they seem to utilize similar mechanisms for controlling the coupling to and activation of the G proteins.

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