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RESEARCH PAPER

Agonist-dependent consequences of proline to alanine substitution in the transmembrane helices of the calcitonin receptor

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Background and purpose: Transmembrane proline (P) residues in family A G protein-coupled receptors (GPCRs) form functionally important kinks in their helices. These residues are little studied in family B GPCRs but experiments with the VPAC1 receptor and calcitonin receptor-like receptor (CL) show parallels with family A receptors. We sought to determine the function of these residues in the insert negative form of the human calcitonin receptor, a close relative of CL.
Experimental approach: Proline residues within the transmembrane domains of the calcitonin receptor (P246, P249, P280, P326, P336) were individually mutated to alanine (A) using site-directed mutagenesis. Receptors were transiently transfected into Cos-7 cells using polyethylenimine and salmon and human calcitonin-induced cAMP responses measured. Salmon and human calcitonin competition binding experiments were also performed and receptor cell-surface expression assessed by whole cell ELISA.
Key results: P246A, P249A and P280A were wild-type in terms of human calcitonin-induced cAMP activation. P326A and P336A had reduced function (165 and 12-fold, respectively). In membranes, human calcitonin binding was not detectable for any mutant receptor but in whole cells, binding was detected for all mutants apart from P326A. Salmon calcitonin activated mutant and wild-type receptors equally, although B_{max} values were reduced for all mutants apart from P326A.
Conclusions and Implications: P326 and P336 are important for the function of human calcitonin receptors and are likely to be involved in generating receptor conformations appropriate for agonist binding and receptor activation. However, agonist-specific effects were observed , implying distinct conformations of the human calcitonin receptor.
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Abbreviations: CT, calcitonin; CGRP, CT gene-related peptide; CL, CT receptor-like receptor; GPCR, G protein-coupled receptor; RAMP, receptor activity-modifying protein

Introduction

Calcitonin, a 32 amino-acid peptide involved in bone homoeostasis interacts with the family B (secretin-like) G protein-coupled receptor (GPCR), the calcitonin receptor (Sexton *et al*., 1999). This receptor forms part of a broader subfamily encompassing amylin, calcitonin gene-related peptide (CGRP) and adrenomedullin receptors (Poyner *et al*., 2002). While calcitonin selectively binds to its namesake receptor or its splice variants, the other pharmacologically defined receptors are heteromeric and are composed of either the calcitonin receptor or the calcitonin receptor-like receptor (CL) in association with receptor activity-modifying proteins (RAMPs) (Poyner *et al*., 2002).

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Transmembrane helices in GPCRs often contain several bends, most of which occur at proline residues. Such distortions in the helices appear to be functionally important; creating movement in the helix, a necessary step in receptor activation (Gether, 2000; Palczewski *et al*., 2000). In family A (rhodopsin-like) GPCRs, six transmembrane helix kinks are predicted (Yohannan *et al*., 2004). The function of many of these kink-forming residues has been investigated. For example, simulations have indicated that the movement of transmembrane helical domain 6 (TM6) upon β_2-adrenoceptor activation is the result of a change in the kink angle of the conserved proline (Gether *et al*., 1997). In the human IP prostanoid receptor, proline to alanine substitution of residues in TM6, in particular, resulted in a loss of receptor function (Stitham *et al*., 2002).

In contrast, only three kinked helices are predicted in family B GPCRs, whereas there are multiple kinks in MRG8 (family C) receptors (Yohannan *et al*., 2004). The differences

in these kink patterns mirrors the fact that signature sequences such as the DRY motif in family A are not found in family B receptors. As such, detailed knowledge of the role of individual residues is required for each receptor family and few assumptions can be made regarding structure-function characteristics. At the present time, rhodopsin is the only GPCR template for modelling and thus ascertaining the true function of residues predicted to be important for structural features in other families of receptor is crucial for interpreting and refining models of them based on this structure.

Proline residues in the transmembrane domains of the two family B GPCRs that have been studied were shown to profoundly influence receptor function. In the vasoactive intestinal peptide (VIP)/pituitary adenylate cyclase-activating peptide (VPAC) 1 receptor, alanine substitution of each individual proline residue resulted in a marked reduction in B_{max} with only small changes in VIP affinity; receptor function was either enhanced or reduced, depending on the position of the mutation (Knudsen *et al*., 2001). In CL, a component of the $CGRP_1$ receptor, a different pattern was observed; cell surface expression was not affected but the binding and function of two proline to alanine mutants was reduced, most noticeably for P321A in TM6 (Conner *et al*., 2005).

The human calcitonin receptor shares 55% amino-acid sequence identity with CL and is its closest relative (Figure 1). Consequently, it could be predicted that the conserved transmembrane proline residues in these two receptors would have similar functions. Therefore, in this study, we sought to identify the functional importance of proline residues situated in TM 4, 5 and 6 of the calcitonin receptor, comparing that with earlier data for CL (Conner *et al*., 2005) in order to help determine whether these residues have family-wide significance, as they do in family A.

P326A and P336A affected calcitonin receptor function with human but not salmon calcitonin. The data suggest agonist-specific conformations of the human calcitonin receptor, of which those that preferentially bind salmon calcitonin are affected to a lesser degree by the removal of proline than those that bind human calcitonin.

Methods

DNA constructs

The hemagglutinin (HA)-tagged human calcitonin receptor (insert negative form ($CT_{(a)}$), leucine polymorphic variant) was kindly provided by Professor PM Sexton (Monash University, Melbourne, Australia) (Pham *et al*., 2004). We have started numbering of amino acid residues after the predicted signal sequence (Figure 1). Human RAMP1 was a gift from Steven M Foord (Glaxosmithkline, Stevenage, UK).

Site-directed mutagenesis

Forward and reverse oligonucleotide primers were designed with single base changes to incorporate amino-acid point mutations from proline to alanine in the calcitonin receptor protein. The mutation was designed to be approximately central to the primer. All primers were custom synthesized by Invitrogen Corporation, (Carlsbad, CA, USA). Forward primers were; P246A: ctt ggg ctg ggg gtt cgc gct ggt gcc aac, P249A: gtt ccc gct ggt ggc aac cac tat cca tg, P280A: cat aat cca tgg agc tgt cat ggc ggc ac, P326A: cat gat cct tgt ggc cct gct ggg aat c, P336A: cag ttt gtc gtc ttt gcc tgg aga cc. For generation of individual mutants, the following were mixed in a 0.5 ml PCR-tube and placed in a thermal cycler; 100 ng plasmid containing calcitonin receptor, 1 μl (5 pmol) of each forward and reverse primers, 5 μl of $10 \times$ *pfu* polymerase buffer, 1 μl 40 mM dNTPs, sterile distilled H_20 to a volume of 49 μl and finally, 1 μl *pfu* DNA polymerase (Promega Corporation, Madison, WI, USA). The following protocol

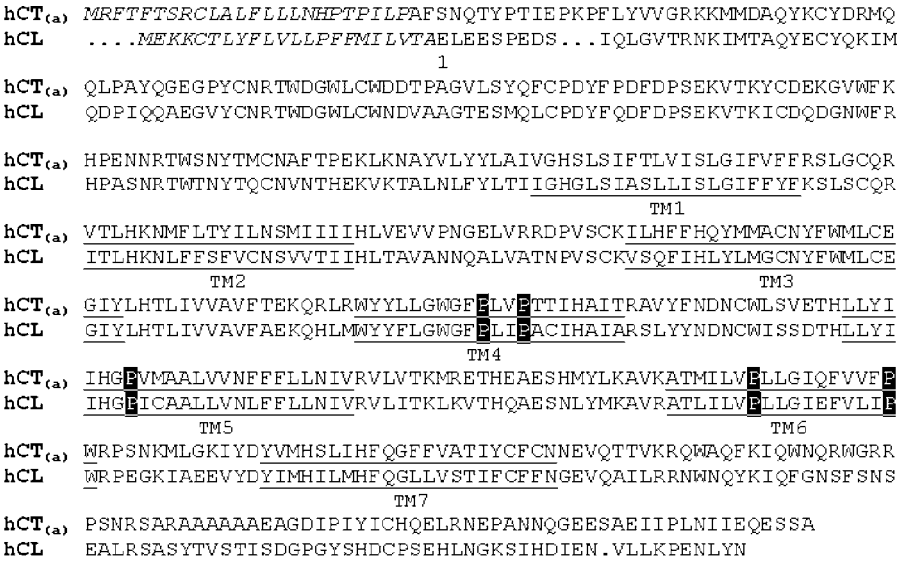

Figure 1 Alignment of the insert negative form of the human calcitonin receptor ($CT_{(a)}$) with its closest relative, the CL showing the conserved transmembrane proline residues in white text on black. Alignment was performed by ClustalW. Predicted signal sequences are italicized, transmembrane (TM) regions are underlined.

was followed: (1) denaturing temperature 95°C 30 s, cycled once, (2) denaturing temperature 95°C 30 s, (3) annealing temperature 55°C 60 s, (4) extension temperature 68°C 530 s (120 s per kb) and (5) steps (2) to (4) were cycled 12 times. When the programme was complete, 1 μl (10 U) of *Dpn*I restriction enzyme (Promega) was added and incubated at 37°C for 1 h to digest completely the methylated (non-mutated template) plasmid. The reaction mixture (2 μl) was taken and transformed into 50 μl of *Escherichia coli* XL-10 Gold ultracompetent cells according to the manufacturers protocol (Stratagene Corporation, La Jolla, CA, USA) and plasmid DNA prepared from the transformed *E. coli*. Mutations were confirmed by sequencing.

Cell culture and transfection

Culture of Cos 7 cells was performed as described (Bailey and Hay, 2006). Briefly, cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat inactivated foetal bovine serum (FBS) and 5% v/v penicillin/streptomycin and kept in a 37°C humidified 95% air/5% CO_2 incubator. Cells were plated into 24- or 96-well plates or T175 cm^2 flasks 1 day before transfection. Cells were transfected using polyethylenimine (PEI) as described (Bailey and Hay, 2006). Briefly, plasmid DNA (250 ng per well of 96-well plate unless otherwise stated; 1 μg per well of 24-well plate, 40 μg per flask) was incubated with PEI, in 5% glucose for approximately 10 min before being added to complete growth medium. Cells were used for experimentation 36–48 h later.

cAMP assay

Cyclic adenosine 3′,5′ monophosphate (cAMP) assays were performed as described previously (Bailey and Hay, 2006). Transfected cells were serum-deprived in DMEM containing 1 mM isobutylmethylxanthine (IBMX) and 0.1% bovine serum albumin (BSA) for 30 min before addition of agonists and incubation at 37°C for 15 min. cAMP was extracted with ethanol and measured by radio-receptor assay.

Membrane preparation

For membrane preparation, Cos-7 cells were grown and transfected in T175 cm^2 tissue culture flasks. On the day of harvest, transfection media were removed and the cells washed twice with ice-cold phosphate-buffered saline (PBS). Ice-cold membrane harvest buffer (50 mM HEPES, 1 mM ethylenediaminetetraacetic acid, pH 7.5) was added (5 ml per flask) and the cells removed using a cell scraper. The dislodged cells were placed on ice, while any remaining cells were rinsed from flasks with a further 2.5 ml ice-cold membrane harvest buffer. The cells were homogenized with an electric homogenizer (Ika–Werke Ultra-Turrax T8 on setting number 6) for four 20 s periods, placing the tube back on ice for 15 s in-between homogenizations. The homogenate was centrifuged in a Sorvall SS34 rotor at 48 000 *g* (20 000 r.p.m.) at 4°C for 1 h and the supernatant discarded. The pellet was re-suspended in 5 ml of ice-cold membrane harvest buffer and re-homogenized. A small aliquot was retained for protein content assay (Bio-Rad D_C protein assay using reagents A and B). The protein yield was typically in the range of 1.2–1.5 mg ml^{-1}. The remainder was frozen at −80°C.

Radioligand binding

Membranes. All binding assays were performed in duplicate in siliconized microcentrifuge tubes (Bio Plas Inc., San Rafael, CA, USA). For human calcitonin binding, a dilution series of non-radio-labelled human calcitonin was prepared in binding buffer (serum-free DMEM with 0.1% BSA) giving final concentrations ranging from 10^{-6} (nonspecific binding) to 10^{-13} M. (3-[^{125}I]iodotyrosyl^2) human calcitonin (^{125}I-hCT, Amersham Biosciences Corporation, Piscataway, NJ, USA) was diluted with binding buffer to allow approximately 20–30 000 c.p.m. in 50 μl ($\sim$14 pM). Membranes were thawed on ice and re-homogenized immediately before use with a glass homogenizer. Diluted radiolabel, appropriate unlabelled competitor from the dilution series or buffer (total binding) and 100 μg membranes were added to each assay tube in this order. The mixture was vortexed briefly and incubated at 37°C for 1 h. The membranes were pelleted in a bench-top microcentrifuge at 4°C for 5 min. The supernatants were carefully removed and the pellets washed once with 500 μl ice-cold PBS before being counted. Salmon calcitonin binding was performed as for human calcitonin with minor modifications. Radioligand was (3-[^{125}I]iodotyrosyl^2) salmon calcitonin (^{125}I-sCT, Amersham, $\sim$7 pM), competing peptide was 10^{-6} to 10^{-13} M salmon calcitonin and the binding buffer additionally contained 5 mM $MgCl_2$.

Whole cells. For human calcitonin binding in whole cells, transfected cells were washed once with binding buffer (serum-free DMEM with 0.1% BSA) and radiolabelled or non-radiolabelled human calcitonin added to the cells in the amounts and volumes described above. The plates were incubated at 37°C for 1 h before being washed once with ice-cold binding buffer. Cells were lysed with 0.1 M NaOH and lysates counted for radioactive content. Some wells were set aside to determine protein content.

Cell-based ELISA

This method is based on that of Versteeg *et al*. (2000) with modifications. One hundred microlitres of 8% paraformaldehyde in 0.1 M phosphate buffer was added directly to each well of a 96-well plate containing transfected cells and transfection media and incubated at room temperature with gentle shaking for 20 min, the wells aspirated and washed twice. All washing was performed with 100 μl PBS for 10 min with gentle shaking. PBS (100 μl) containing 0.6% hydrogen peroxide was added to each well and incubated at room temperature with gentle shaking for 20 min and then washed once. PBS (100 μl) with 10% goat serum was added to block nonspecific binding and incubated at room temperature with gentle shaking for 1 h. The wells were aspirated and 50 μl of anti-HA.11 monoclonal primary antibody (Covance: MMS-101P, diluted 1:2000 in PBS with 1% goat serum) was added and incubated for 30 min at 37°C before being washed once. Horseradish peroxidase-linked anti-mouse secondary

antibody (Amersham: NA931-1ML, diluted 1:500 in PBS with 1% goat serum) was added and incubated at room temperature with gentle shaking for 1 h. The wells were aspirated and washed twice. Substrate was SIGMAFAST OPD (Sigma-Aldrich Corporation, St Louis, MS, USA) and was incubated for 15 min in the dark with gentle shaking. The reaction was stopped by adding 0.5 M H_2SO_4, mixing gently and plates were read at A_{490} and A_{650}. After two washes, cresyl violet working solution (250 μl 1% cresyl violet solution plus 9 ml 0.6% glacial acetic acid and 1 ml of 25 mM sodium acetate) was added to each well and incubated at room temperature with gentle shaking for 30 min. The wells were washed once and 1% sodium dodecyl sulphate (SDS) added and incubated at room temperature with gentle shaking for 1 h. The A_{595} was determined and the $(A_{490}-A_{650})/A_{595}$ calculated for each well. Values were background-corrected and then normalized to wild-type expression levels.

Western blotting

Membrane protein (10 μg) was heated with NuPage LDS Sample Buffer (Invitrogen) containing 100 mM DL-dithiothreitol (Sigma) for 30 min at 60°C and loaded onto a NuPage 4–12% Bis-Tris gel (Invitrogen) and electrophoresed for 35 min at 200 V with NuPage MES SDS running buffer (Invitrogen). A BenchMark Pre-stained Protein Ladder (Invitrogen) and Magicmark XP Western Standard (Invitrogen) were also included. The protein was transferred to polyvinylidene difluoride (PVDF) membrane by blotting for 1 h at 30 V with NuPage Transfer Buffer (Invitrogen). The PVDF membrane was then blocked for 30 min using 5% dried skimmed milk powder in PBS-0.2% Tween (PBS-T). The blocked membrane and primary antibody (anti-HA, as above) were incubated for 1 h at room temperature with gentle shaking and washed. All washing was performed twice in an excess of PBS-T with gentle shaking for 5 min. The membrane was then incubated for 30 min with anti-mouse secondary antibody (1:5000, as above) and washed. Detection was performed using ECL Plus and Hyperfilm ECL (Amersham).

Data analysis and statistical procedures

Data were analysed using Graphpad Prism (version 4.02). cAMP data were first normalized to the response obtained to 50 μM forskolin that was present as a control on each plate or cAMP concentrations in each well back-calculated from a cAMP standard curve. Binding and cAMP data were fitted to obtain pIC_{50} and pEC_{50} values, respectively. B_{max} values were calculated from human calcitonin whole-cell and salmon calcitonin membrane homologous competition-binding curves. K_i values were calculated from IC_{50} values using the Cheng Prusoff equation. K_i was assumed to be the same as the K_d. This was used to determine B_{max} values by dividing the specific binding by the fractional occupancy.

For statistical analysis, B_{max}, pIC_{50} or pEC_{50} values were compared between wild-type and mutant receptors using one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test where appropriate. E_{max} values were compared using Kruskal–Wallis or one-way ANOVA. Significance was achieved at $P<0.05$.

Drugs, chemicals and other materials

Salmon and human calcitonin were purchased from Bachem (Bubendorf, Switzerland) and rat amylin was from Auspep; peptides were dissolved in water to make 1 mM stock solutions (taking into account peptide content) and stored as aliquots at −30°C in siliconized microcentrifuge tubes. IBMX, protein kinase A and activated charcoal were from Sigma. DMEM and FBS were from Invitrogen. Forskolin was from Tocris. All other reagents were of analytical grade.

Results

Stimulation of cAMP production in response to human and salmon calcitonin

Analysis of human calcitonin-stimulated cAMP production by mutant calcitonin receptors revealed that P246A, P249A and P280A did not behave significantly differently to the wild-type receptor; pEC_{50} values were equivalent (Table 1, Figure 2a–c). On the other hand, the proline to alanine substitutions in TM6; P326A and P336A, respectively, resulted in significantly lower potency stimulation of cAMP by human calcitonin than wild-type receptors (Table 1, Figure 2d and e). The substitution at position 326 reduced agonist potency by approximately 165-fold, whereas at position 336 the substitution was not as detrimental to function with only about a 12-fold loss. Full efficacy was retained at all mutant receptors (Table 1).

A second agonist of calcitonin receptors, salmon calcitonin was also tested at mutant calcitonin receptors and its effects compared with wild-type receptors. Unexpectedly and in marked contrast to the human calcitonin data, salmon calcitonin was found to retain high potency and efficacy equivalent to wild-type at all mutant receptors (Table 1).

Stimulation of cAMP production by rat amylin in the presence of RAMP1

As calcitonin receptors interact with RAMPs to form amylin receptors, wild-type and mutant calcitonin receptors were co-transfected with RAMP1 to compare the impact of the mutations in the presence of RAMP. Rat amylin-stimulated cAMP production at wild-type or mutant $AMY_{1(a)}$ receptors generated a similar pattern of receptor perturbation to that seen with human calcitonin. This was ~50-fold loss of rat amylin potency at P326A and ~5-fold at P336A. The pEC_{50} for rat amylin at wild-type $AMY_{1(a)}$ receptors was 10.15 ± 0.10 ($n=4$) compared with 10.09 ± 0.10 ($n=4$) at P246A, 9.90 ± 0.17 ($n=4$) at P249A, 9.71 ± 0.16 ($n=4$) at P280A, 8.44 ± 0.17 ($n=4$, $P<0.01$ vs wild-type by one-way ANOVA) at P326A and 9.45 ± 0.14 ($n=4$, $P<0.05$ vs wild-type by one-way ANOVA) at P336A $AMY_{1(a)}$ receptors.

^{125}I-hCT and ^{125}I-sCT binding

Homologous competition binding curves were constructed for both human and salmon calcitonin at all receptors. In membrane-binding experiments, human calcitonin in competition with ^{125}I-hCT produced biphasic curves, which

Table 1 pEC_{50}, E_{max}, pIC_{50} and B_{max} data for WT and mutant calcitonin receptors, with hCT or sCT as agonists

	hCT				*sCT*				*hCT*					*sCT*		
									Membrane			*Whole cell*				
	pEC_{50}	E_{max}	n	n_H	pEC_{50}	E_{max}	n	n_H	pIC_{50}	n	pIC_{50}	B_{max} (fmol mg^{-1})	n	pIC_{50}	B_{max} (fmol mg^{-1})	n
WT	10.62±0.29	77.8±3.1	9	0.45[a]	11.16±0.24	92.4±4.1	5	0.54[a]	7.94±0.12(0) 11.2±0.31(h)	9	8.13±0.06	4642±637	3	9.22±0.04	1072±83	3
P246A	10.15±0.24	65.4±6.0	5	0.57	11.35±0.12	85.5±2.5	4	0.81	NS	3	8.58±0.34	1489±1358*	3	9.54±0.69[b]	287±254[b]	2
P249A	10.09±0.20	65.1±1.4	4	0.38[a]	11.42±0.11	90.0±1.6	3	0.72[a]	NS	3	8.54±0.17	538±177*	3	9.37±0.04	168±35**	3
P280A	10.10±0.28	73.6±0.94	4	0.56[a]	11.37±0.05	87.3±1.1	3	0.71[a]	NS	3	9.10±0.15*	60±26**	3	9.83±0.36	69±30**	3
P326A	8.40±0.38**	66.3±4.5	4	0.53	11.08±0.38	92.9±5.1	4	0.54[a]	NS	3	NS	NS	3	9.23±0.05	600±70	3
P336A	9.52±0.19*	76.1±4.8	4	0.61	11.67±0.21	91.1±2.5	3	0.67[a]	NS	3	8.75±0.27[b]	57±9[b]	2	9.42±0.12	543±258*	3

Abbreviations: h, high affinity site; hCT, human calcitonin; l, low affinity site; NS, insufficient specific binding to generate curves; sCT, salmon calcitonin; WT, wild type.
*$P<0.05$, **$P<0.01$ vs WT one-way ANOVA followed by Dunnett's multiple comparison test.
E_{max} (% forskolin) values compared using Kruskal–Wallis test.
[a]Hill slope different from unity by F-test.
[b]Mean is for two experiments, binding in a third was too low to generate values.

could clearly be differentiated into high and low affinity sites (Table 1, Figure 3a). Conversely, we were not able to detect significant specific binding for any of the receptor mutants in membranes prepared from three separate transient transfections. Figure 3a shows an example of data generated for the P246A mutant. The calcitonin receptor construct used in this study is HA-tagged so, to confirm that receptors were present in these membrane preparations, western blotting was performed. All membranes tested showed that HA-tagged receptors were present (data not shown). Human calcitonin did not appear to be able to bind to any of the mutant receptors, but in earlier experiments (Table 1, Figure 2a–c), we had shown that three of these mutants behaved equivalently to wild-type receptors in terms of cAMP production. Therefore, we speculated that the mutant receptors may have been particularly sensitive to the loss of stabilizing factors in the process of preparing the membranes.

Binding experiments were therefore repeated in whole cells. For wild-type receptors, in contrast to membrane binding, a single site fit was most appropriate for the data generated, the pIC_{50} being similar to the low affinity value generated for two site analysis with the membrane binding data (Table 1, Figure 3e). Interestingly, human calcitonin binding was detectable for four out of five proline mutants under these conditions, although B_{max} values were considerably lower than for the wild-type receptor (Table 1, Figure 3e and f). P326A produced very low-level binding that was not sufficient to generate IC_{50} or B_{max} values (not shown). pIC_{50} values for mutant receptors were not reduced compared with the wild-type calcitonin receptor, although there was an apparent 10-fold increase in human calcitonin affinity at P280A (Table 1).

We also investigated salmon calcitonin binding using membranes. At wild-type calcitonin receptors, the salmon calcitonin-binding curve best fitted a single site (Figure 3b). In contrast to the ^{125}I-hCT membrane binding data, we were able to generate binding curves to ^{125}I-sCT at each of the mutant receptors. pIC_{50} values for P249A, P280A, P326A and P336A were not significantly different to the wild-type receptor (Table 1, Figure 3c and d). P246A (Figure 3b) produced variable amounts of binding and in one experiment did not produce sufficient binding to enable generation of a pIC_{50}. When checked by western blotting, these membranes did contain tagged calcitonin receptors (not shown). In two other experiments, pIC_{50} values were comparable with wild-type receptor-expressing membranes (Table 1) but statistical analysis could not be performed on this mutant. Overall, B_{max} values were lower at all mutant receptors, although this did not reach significance for P326A (Table 1).

Cell-surface expression

The effect of proline to alanine substitution on the cell-surface expression of the receptors was evaluated using a whole-cell enzyme-linked immunosorbent assay (ELISA) based method. HA-tag was detected at the cell surface and compared across receptors. As shown in Figure 4, three of the mutants, P246A, P249A and P280A, appeared to express at a

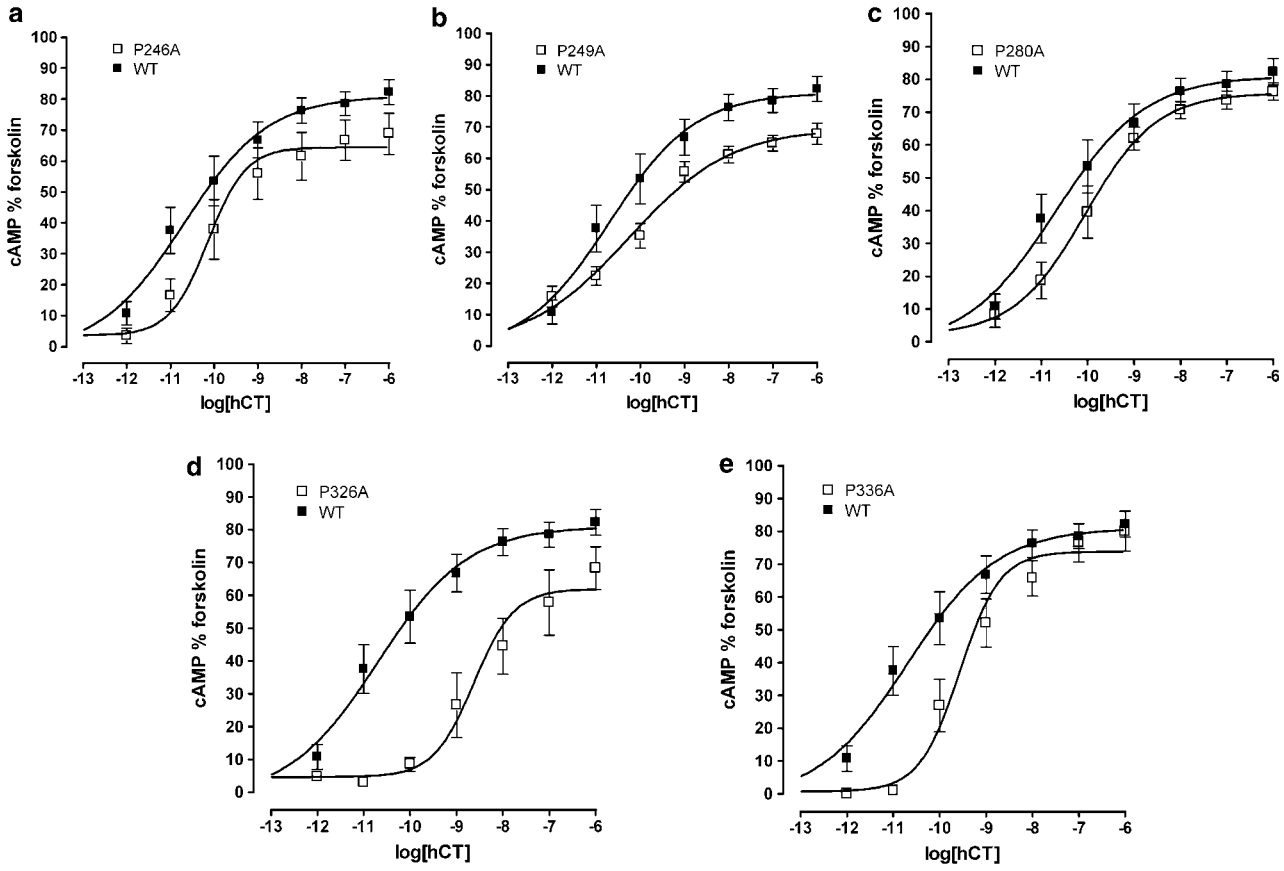

Figure 2 Stimulation of cAMP production by human calcitonin at calcitonin receptor proline mutants or wild-type (WT) calcitonin receptors, as indicated (**a**–**e**) expressed as a percentage of the cAMP response generated by 50 μM forskolin. Data are mean$\pm$s.e.m. of four to nine experiments, performed in duplicate or triplicate.

lower level than wild-type receptors, although this did not reach significance for P249A.

Effect of varying DNA quantity on cAMP responses

We hypothesized that there may have been sufficient receptor reserve in our system that binding and expression could be significantly reduced without observing changes to potency. We tested this by comparing cAMP responses at wild-type and P246A receptors in cells transfected with different amounts of receptor DNA (empty vector was co-transfected to ensure the total amount remained the same in all groups). As shown in Table 2 and Figure 5, progressive reductions in DNA quantity caused reductions in E_{max} at both receptors, whereas pEC_{50} values were unchanged. Up to a fourfold reduction in DNA quantity did not significantly alter potency or E_{max}. Even at 1/16th of the amount of DNA usually used, clear curves were still present.

Discussion and conclusions

Transmembrane proline residues play a critical role in the fluid movement of GPCR transmembrane helices, a critical step in receptor activation. In family A receptors, the functionality of individual proline residues has been extensively studied. In contrast, in family B GPCRs only the VPAC1 receptor (Knudsen *et al.*, 2001) and more recently, CL (Conner *et al.*, 2005) transmembrane prolines have been studied and therefore it is only an assumption that prolines fulfil a similar role in this GPCR family. Broadly, proline to alanine substitution of some of the residues resulted in altered receptor function, although the precise nature of the alteration was receptor dependent. In this study, we sought to determine the role of the transmembrane proline residues situated in the transmembrane helices of the calcitonin receptor, the closest relative of CL to contribute towards deciphering the function of these residues in family B GPCRs.

Mutation of proline residues at positions 246, 249 and 280 to alanine did not measurably alter receptor function when human calcitonin was used as agonist, consistent with data generated for residues in the same position in CL (Conner *et al.*, 2005). On the other hand, mutation of residues at positions 326 and 336 resulted in reduced function, approximately 165-fold for P326A and 12-fold for P336A. This compares favourably with the loss of function observed with mutation of the equivalent residues in CL, 200-fold for P321A (326 equivalent) and 10-fold for P331A (336 equivalent). Investigation of ^{125}I-hCT membrane binding revealed a different pattern; binding was lost in all mutants. This was unexpected given that function was apparently preserved in

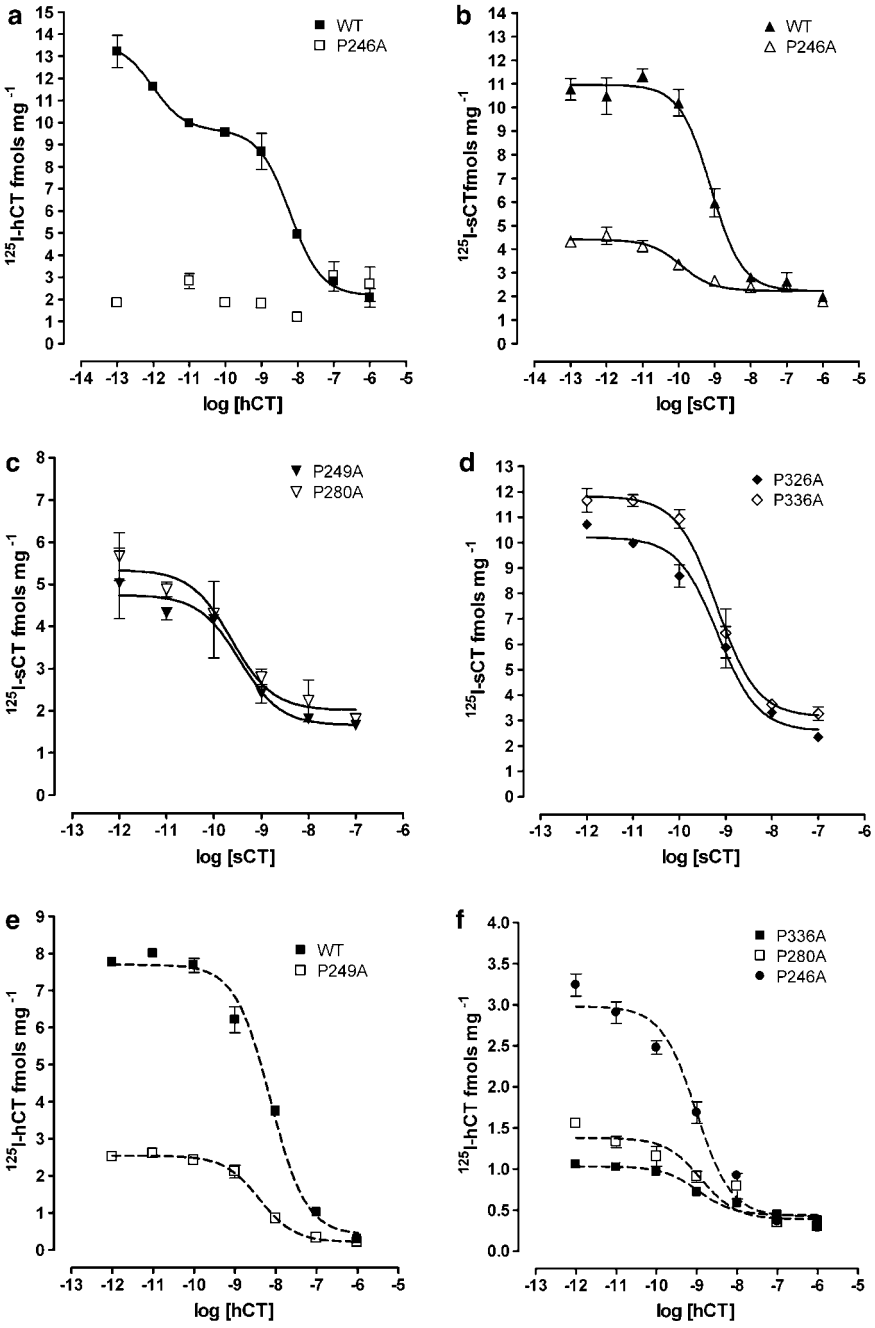

Figure 3 Binding of ^{125}I-hCT (**a**) or ^{125}I-sCT (**b**, **c**, **d**) to membranes or ^{125}I-hCT (**e**, **f**) binding to cells transiently transfected with wild-type (WT) or mutant calcitonin receptors. Experiments were repeated three to nine times. Data shown are representative and data points are mean ± s.e.m. of duplicate or triplicate points.

three out of five mutants. Membranes used in the binding studies were tested by western blotting and shown to express receptor, indicating that the lack of binding was not due to transfection failure. Therefore, binding in whole cells was investigated in case the membrane preparation process resulted in an artefactual loss of human calcitonin binding at mutant receptors. Indeed, this appeared to be the case as binding could be detected at most of the mutant receptors under whole-cell binding conditions albeit at lower levels than wild-type receptors. On the other hand, binding was still not detectable for P326A, the mutant that had the greatest loss of functionality in response to human calcitonin. There was no reduction in binding affinity for the remaining mutants, although there was an apparent 10-fold increase in affinity at P280A.

Investigation of receptor expression by whole-cell ELISA indicated that P326A and P336A were present at the cell surface at equivalent levels to wild-type receptors. For these mutants, a reduction in human calcitonin interactions did not appear to be due to a change in cell surface expression. In contrast, P246A, P249A and P280A expression was reduced by about half, although this did not reach significance for P249A. Here,

there was a reduction in B_{max} and expression without any change in potency. Changes in B_{max} for these mutants might be related, at least in part, to reduced expression levels.

A point of note is that there appeared to be considerable receptor reserve in our system. Only relatively few receptors were required to elicit a full response; in many cases, B_{max} and expression were reduced but functional response maintained. For example, in expression studies, P246A levels were reduced by about half. However, when the DNA concentration was halved, full activity in response to human calcitonin was retained. In our receptor expression (ELISA) assay, a twofold reduction in calcitonin receptor DNA halves the detected signal (data not shown). Together, this suggests that half the receptors can be lost without affecting potency or maximum response.

The extracellular loops and N-terminal domain of the calcitonin receptor are thought to be the primary site of interaction for peptide ligands in common with other family B GPCRs (Dong *et al*., 2004; Pham *et al*., 2004, 2005; Hoare, 2005). In this study, we observed that single proline to

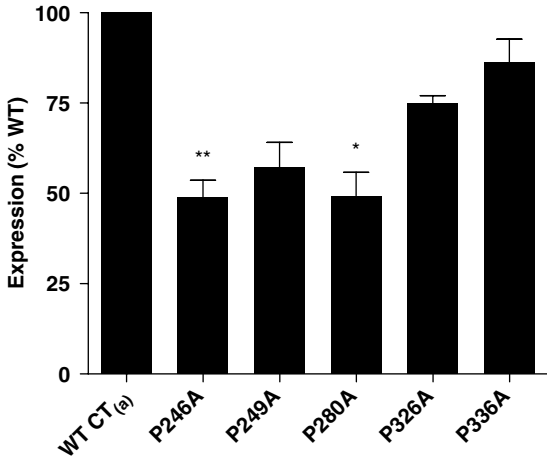

Figure 4 Cell-surface expression of HA-tagged wild-type (WT) or mutant calcitonin receptors. Mutant expression was normalized to WT expression and combined data are shown. Experiments were performed in quadruplicate and data shown are the mean ± s.e.m. of three to four separate experiments. *$P<0.05$, **$P<0.01$ compared to WT by non-parametric one-way ANOVA (Kruskal–Wallis test) followed by Dunn's multiple comparison test.

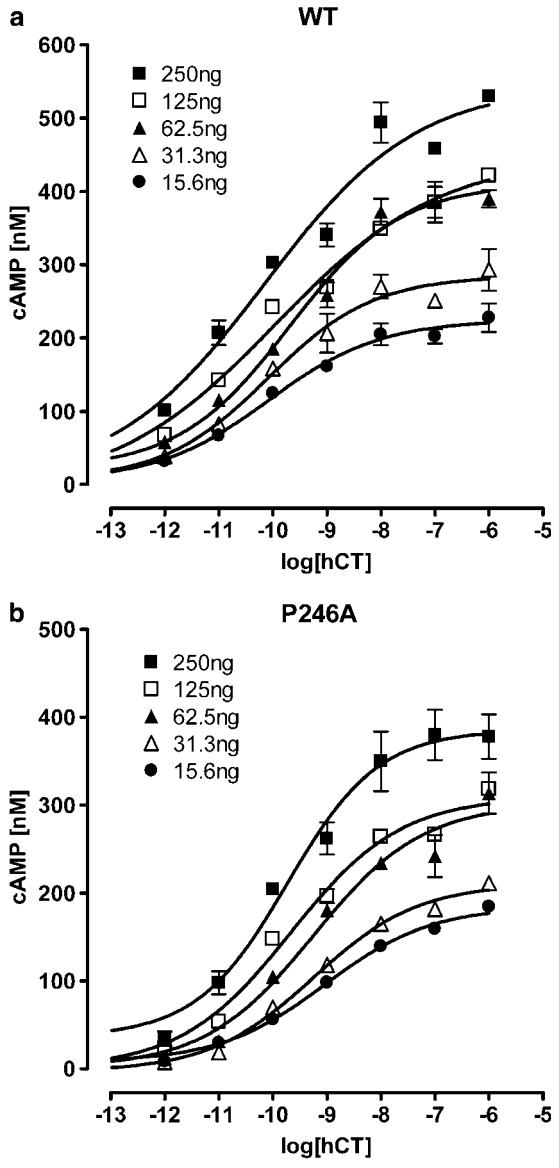

Figure 5 Effect of DNA dilution on human calcitonin-stimulated cAMP production at wild-type (WT) (**a**) or P246A (**b**) calcitonin receptors. The legends show the amount of receptor DNA transfected into each well. Experiments were repeated three times. Data shown are representative and data points are mean ± s.e.m. of duplicate points.

Table 2 The effect of different amounts of WT or P246A receptor DNA on function (pEC_{50} and E_{max})

	WT			*P246A*		
	pEC_{50}	E_{max}	n	pEC_{50}	E_{max}	n
250 ng	9.96 ± 0.10	363.7 ± 91.0	3	10.03 ± 0.42	485.0 ± 59.2	3
125 ng	9.64 ± 0.21	314.0 ± 64.0	3	9.80 ± 0.12	391.4 ± 44.2	3
62.5 ng	9.61 ± 0.04	271.2 ± 71.6	3	9.60 ± 0.18	347.1 ± 22.8*	3
31.3 ng	9.62 ± 0.24	205.4 ± 39.9**	3	9.39 ± 0.26	273.2 ± 36.4**	3
15.6 ng	9.58 ± 0.32	168.8 ± 28.2**	3	9.28 ± 0.59	214.6 ± 15.5**	3

Abbreviations: WT, wild type.
*$P<0.05$, **$P<0.01$ vs WT 250 ng or P246A 250 ng as appropriate by one-way ANOVA followed by Dunnett's multiple comparison test.
E_{max} values are the maximum asymptote of concentration–effect curves and are concentrations (nM) of cAMP.

alanine substitutions in the transmembrane domains of the calcitonin receptor resulted in a reduction in human calcitonin binding. In the VPAC1 receptor, it was reported that binding was not affected by substitution of transmembrane proline residues with alanine. However, the assay used to detect receptor expression was, in fact, a competition binding assay and while it is apparent that binding affinity did not change, the amount of binding detected was reduced in all mutants (Knudsen *et al.*, 2001). When DNA levels were increased, expression (B_{max}) returned to wild-type levels. Thus, agonist binding probably was affected in this study and is consistent with the data presented here. At CL, agonist (CGRP)-binding affinity was only substantially reduced by mutation of the residue, which is equivalent to P326 in the calcitonin receptor (Conner *et al.*, 2005). There were no significant changes in B_{max}. Interestingly, a truncated form of CGRP, which is an antagonist ($CGRP_{8-37}$) bound normally suggesting that the proline residue in this position was important for the integrity of a receptor conformation, which binds agonists. We did not have access to an antagonist radioligand to test this for the calcitonin receptor but in terms of reduced human calcitonin receptor-stimulated cAMP activity, P326 in the calcitonin receptor may have a similar function to the equivalent residues in CL. Thus, certain proline residues in the transmembrane domains of the calcitonin receptor may be critical for the appropriate formation of receptor conformations that bind agonists, whether agonist binding is best explained by conformational selection or induced fit. It is worth noting that a two-step-binding mechanism has been proposed for the interaction of peptide ligands with family B GPCRs (Castro *et al.*, 2005; Hoare, 2005). Reduced flexibility in the receptor structure, owing to the loss of proline, is likely to impair this process and hence agonist interactions. Whatever the mechanism of agonist binding, it is likely that the structural change induced by removal of individual prolines modifies either the flexibility or inherent shape of TM6 such that its relationship with the other helices and therefore extracellular loops modifies the interactions of human calcitonin. It is known that changes to the extracellular loops of calcitonin receptors can change binding kinetics. For example, the rat C1b receptor has a 37 amino acid insert in the first extracellular loop; this insert changes the kinetics of ^{125}I-sCT binding, resulting in improved dissociation of the radioligand compared with the rat C1a receptor, which does not have this insert (Houssami *et al.*, 1994). We speculate that a subtle change in the orientation of TM6 could have a secondary effect on the positioning of the other receptor helices leading to modified juxtamembrane and loop presentation to a ligand, thus modulating its interactions.

A second agonist of calcitonin receptors, salmon calcitonin was also tested. In contrast to the human calcitonin cAMP data, receptor function was preserved in all mutants. In CL, there was no apparent difference in the behaviour of proline mutants with agonist; both CGRP and adrenomedullin behaved similarly at each mutant (Conner *et al.*, 2005). Unlike ^{125}I-hCT binding, ^{125}I-sCT membrane binding was detectable in all mutants, consistent with the cAMP data for this agonist. Interestingly, B_{max} values were reduced for all mutants apart from P326A. This may be due to decreased expression for P246A, P280A and possibly P249A. Reductions in B_{max} for P246A, P249A and P280A did not result in any change in salmon calcitonin affinity or potency, consistent with data for these mutants when human calcitonin was used. On the other hand, there were differences in B_{max} for P326A and P336A for the two agonists used. This was most noticeable for P326A where there was no significant reduction in B_{max}, affinity or potency for salmon calcitonin but no B_{max} could be obtained for human calcitonin and potency was significantly affected by this mutation.

Salmon calcitonin is known to bind essentially irreversibly to calcitonin receptors (Hilton *et al.*, 2000) and this causes persistent activation of cAMP (Michelangeli *et al.*, 1983). Such an avid interaction with the receptors could mask the effect of the proline to alanine substitutions. While salmon and human calcitonin appear to have broadly similar modes of interaction with calcitonin receptors (Dong *et al.*, 2004; Pham *et al.*, 2004), it is conceivable that subtle changes in receptor conformation as might be expected by the removal of proline residues could affect the mode of interaction of one agonist more than the other. Given that salmon calcitonin binds with higher affinity than human calcitonin, the effect of the proline to alanine substitutions on receptor structure may have resulted in greater propensity toward disrupting human calcitonin over salmon calcitonin binding. Interestingly, small modifications to parathyroid hormone (PTH)-related protein, an agonist of the human PTH type 1 receptor, appear to result in distinct conformations of the receptor, based around changes to the movement of TM 5 and 6 (Bisello *et al.*, 2002). Thus, amino acid differences between human and salmon calcitonin could be sufficient to induce distinct calcitonin receptor conformations. There is already some evidence for this in that Gαs supplementation in human embryonic kidney293 cells transfected with the calcitonin receptor increased human calcitonin potency, without altering salmon calcitonin potency (Watson *et al.*, 2000).

The closest relative to the calcitonin receptor is CL and as shown in Figure 1 both of these receptors share the same set of proline residues. However, CL requires RAMPs for function. In order to more accurately compare the consequences of proline to alanine substitution between these two receptors, we transfected our calcitonin receptor proline mutants with RAMP1 to generate $AMY_{1(a)}$ receptors. When stimulated with rat amylin, the resulting changes in agonist potency mirrored those observed with human calcitonin at the calcitonin receptor alone. There were no significant changes with P246A, P249A and P280A but $\sim$50- and $\sim$5-fold reductions in amylin potency were observed for P326A and P336A, respectively.

Overall, the effects of the proline to alanine substitutions in the calcitonin receptor appear to be intermediate between effects at CL and the VPAC1 receptors. Human calcitonin potency was reduced for the same residues in CL and the calcitonin receptor. In the VPAC1 receptor, the P326A equivalent (P348A) increased agonist potency and there is no P336A equivalent in this receptor. On the other hand, B_{max} values were reduced in the mutant calcitonin receptors as they were in the VPAC1 receptor but not in CL. Expression levels were reduced for some calcitonin receptor mutants but there were no changes in expression for CL, although it

should be noted that calcitonin receptor expression studies were performed in the absence of RAMP1, whereas CL studies were with RAMP1. The data presented in this study offer an interesting conundrum; even in closely related receptors, which bind the same family of peptides the effects of proline to alanine substitution are not identical. There are three highly conserved transmembrane proline residues in the family B peptide receptors. These are the equivalents to P246A, P280A and P326A in the human calcitonin receptor. Based on the data available so far for the three family B receptors in which these residues have been studied, it is difficult to predict the consequences of mutating these residues in other family members.

Nonetheless, substituting proline residues with alanine in the transmembrane domains of family B GPCRs, in particular at the P326A equivalent does appear to modify agonist interactions. The data may be consistent with modifications to the movements of TM6, the consequences of which appear to differ between receptors. In the case of the calcitonin receptor, it is apparent that if the agonist has sufficient efficacy, as may be the case for salmon calcitonin, it can overcome the effect of the loss of proline. It is not clear whether the changes to the receptor predominantly affect binding or activation mechanisms. Salmon calcitonin could activate the calcitonin receptor in a different way to human calcitonin and consequently its interactions with the receptor were not affected by the loss of proline. On the other hand, structural modification that affects binding rather than activation could equally well explain the data. The two effects may not be independent of one another and this will require further experimentation to delineate.

In summary, proline residues in the transmembrane helices of family B GPCRs appear to be important for the function of these receptors. However, there is neither sufficient data nor consistency to support a common role for these residues between family A and family B GPCRs. Together, the studies on these residues illustrate that different GPCR families do not necessarily share identical modes of activation and data derived for family A is not necessarily transferable to family B.

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Conflict of interest

The authors state no conflict of interest.

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