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Molecular modelling study of the role of cholesterol in the stimulation of the oxytocin receptor★✪

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Cholesterol, an integral component of membranes in *Eucaryota*, is a modifier of membrane properties. *In vivo* studies have demonstrated that cholesterol can also modulate activities of some G protein-coupled receptors (GPCRs), which are integral membrane proteins. This can result either from an effect of cholesterol on the membrane fluidity or from specific interactions of the membrane cholesterol with the receptor, as recently demonstrated for the cholecystokinin type β (CCKRβ) or the oxytocin receptor (OTR). Using molecular modelling, we studied conformational preferences of cholesterol and several of its analogues. Subsequently, we simulated the distributions of their preferred conformations around the surface of OTR, CCKRβ and a chimeric oxytocin/cholecystokinin receptor. Consequently, we suggest residues on the surface of OTR which are potentially significant in the OTR/cholesterol interaction.

Cholesterol is one of the major components of cellular membranes in *Eucaryota*, modulating mechanical and transport properties of these membranes. For instance, an increase of the cholesterol content in a membrane causes an increase of its elasticity (Méléard *et al.*, 1997) and a decrease of its passive permeability for small molecules (Carruthers & Melchior, 1993; Xiang & Anderson, 1997). In addition, cholesterol rises the stability of membrane proteins (Maneri & Low, 1988). Also, its presence is a requisite for membrane proteins function, e.g. for transferrin (Nunez & Glass, 1982), nicotinic acetylcholine (Narayanaswami & McNamee, 1993), oxytocin

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Abbreviations: CCKR, cholecystokinin receptor; GPCR, G protein-coupled receptor; OTR, oxytocin receptor; TM, transmembrane.

(Klein *et al.*, 1995; Gimpl *et al.*, 1995; 1997) and galanin (Ga1R2) (Pang *et al.*, 1999) receptors, and for rhodopsin (Albert *et al.*, 1996; Boesze-Battaglia & Albert, 1990). There is evidence that cholesterol can modulate the function of membrane receptors in two ways: either *via* changes in membrane fluidity or *via* a highly selective interactions between cholesterol and the protein (Gimpl *et al.*, 1997).

Regarding membrane fluidity, *Procaryota* regulate it *via* a modulation of the contents of unsaturated bonds and the length of the hydrocarbon chains in their membrane lipids, while in *Eucaryota* cholesterol plays a critical role in the regulation of membrane fluidity. By fitting in between the hydrocarbon chains of the lipid bilayer, cholesterol prevents its crystallization, whereas high concentrations of cholesterol extend temperature ranges for phase transitions of membranes. Another effect of cholesterol is hindering the hydrocarbon chains mobility, resulting in a decrease of a membrane fluidity (Stryer, 1995).

Experimental studies have proved that the distribution of cholesterol in the cellular membranes is not random (Liscum & Underwood, 1995; Schroeder *et al.*, 1991) to ensure an adequate structural environment for its specific interactions with membrane proteins. An example may be specific interactions of oxytocin receptor (OTR) with membrane cholesterol, indicated by sigmoidal shape of a radioligand binding curve as a function of membrane saturation with cholesterol (Gimpl *et al.*, 1997). OTR, stimulated with the hypophyseal nonapeptide hormone oxytocin, controls birth labour and lactation in mammals. Another example in the same work, cholecystokinin receptor type β exhibits linear binding curve as a function of membrane saturation with cholesterol, indicating that the CCKRβ ligand affinity is only a function of membrane fluidity (Gimpl *et al.*, 1997).

Both OTR and CCKRβ belong to the superfamily of G protein-coupled receptors (GPCRs), whose activities depend on interactions with GDP-binding (G) proteins on the cytosolic side of the membrane (Sprang, 1997). All GPCRs are composed of a single heptahelical polypeptide chain, utilizing the helices for traversing the phospholipid bilayer 7 times to and fro, starting on the extracellular and ending on the cytosolic side. Thus, they make a heptahelical transmembrane bundle domain (TM), while the associated loops are cores of the extracellular and cytosolic domains, composed of three loops each plus the N- and C-terminal sequence, respectively. In the abundant rhodopsin subfamily, to which both OTR and CCKRβ belong, the TM domains are homologous to 20%. Until very recently (Palczewski *et al.*, 2000), no atomic-resolution GPCR structures were known and only theoretical models, typically but not exclusively based on a low-resolution (to about 6 Å) structure of rhodopsin from electron cryomicroscopy (Schertler & Hargrave, 1995; Unger *et al.*, 1997) were available (Baldwin, 1993; Baldwin *et al.*, 1997; Herzyk & Hubbard, 1995; Peitsch *et al.*, 1995; Pogozheva *et al.*, 1998). Luckily enough, the rhodopsin template we chose in this work (Pogozheva *et al.*, 1998), *vide infra*, fitted the experimental structure of rhodopsin (Palczewski *et al.*, 2000) best of all theoretical models, which manifested in the root-mean-square (r.m.s.) deviation of only 1.71 Å between the transmembrane bundles, contributed mainly by gradual forking of TM helix 5 (TM5) axes to rich about 5 Å at their extracellular ends and by a misfit of TM6 helices by a pitch. Hence, we sustained with our model in this work, despite the landmark recent progress contributed by Palczewski *et al.* (2000).

The details of putative cholesterol–receptor interactions are unknown. In this work we report our attempt to find an area on the surface of the OTR TM domain possibly responsible for interactions between cholesterol and the receptor. To this aim, a distribution of cholesterol and some other selected sterols around an OTR model was simulated. A similar set of simulations was carried out for CCKRβ. Subsequent comparison of both sets of simulations made it possible to select an area on OTR TM domain of potential relevance for the receptor–cholesterol interactions, defined by residues of TM5 and of TM6. Finally, to further test this hypothesis, the same set of simulations was carried out for a mutant receptor, based on the structure of OTR in which the above residues

from TM5 and TM6, as possibly responsible for the OTR–sterol interaction, were replaced with the respective CCKRβ residues.

METHODS

The models of human OTR, CCKRβ and the chimera were obtained using multiple sequence alignment (Corpet, 1988) for the selection of homologous TM helical and loop sequences, see Fig. 1. The alignment comprised four human neurophyseal (OTR and vasopressin V1a, V1b and V2), two human cholecystokinin (CCKRα and CCKRβ) and four human opiate (μ, δ, κ and orphanin) receptor sequences, see Fig. 1. Having the alignment done, OTR and CCKRβ models were subsequently obtained by applying appropriate sets of computer mutations using the human opiate δ receptor (OPRD) model (Pogozheva *et al.*, 1998) as a template, as described elsewhere (Giełdoń *et al.*, 2000). The template itself is an OPRD sequence threaded onto an averaged GPCR structure resulting from a special application of a distance geometry procedure (Pogozheva *et al.*, 1998). Its specialty consisted in the fact that the distance constraints were selected so as to maximize polar interactions *individually* in the interiors of 410 homological GPCRs while simultaneously to converge all their sequences to an averaged *common* backbone trace (Pogozheva *et al.*, 1998). The missing cytosolic loops 2 and 3, likewise the N- and C-terminal sequences, truncated to 10 and 15 residues, respectively, were added using SYBYL 6.6 (1999) molecular modelling package. For the C-termini the truncation site was two residues past the conserved double Cys sequences to allow for each receptor model the putative 4th cytosolic loop. On the other hand, we neglected a majority of the N- and C-terminal residues (from 23 in the V2R N-end to 50 in the V1aR C-end) since their roles, although somewhat obscure, were proven not essential in ligands binding, while their conformation(s) could not be reliably modelled. The starting structures of the receptor models were minimized and relaxed by constrained simulated annealing (CSA) *in vacuo* using the AMBER 5.0 force field (Case *et al.*, 1997). The TM C^{α} positional constraints were set to assure a conservation of a general shape of the receptor while relaxing most of the intramolecular interactions. More details on the CSA protocol are given in Giełdoń *et al.* (2000).

The starting template for all sterols was the solid state structure of cholesterol (Shieh *et al.*, 1981). The conformational space analysis *in vacuo* for the sterols was done using the Monte Carlo conformational sampling as implemented in the PCModel program (1991).

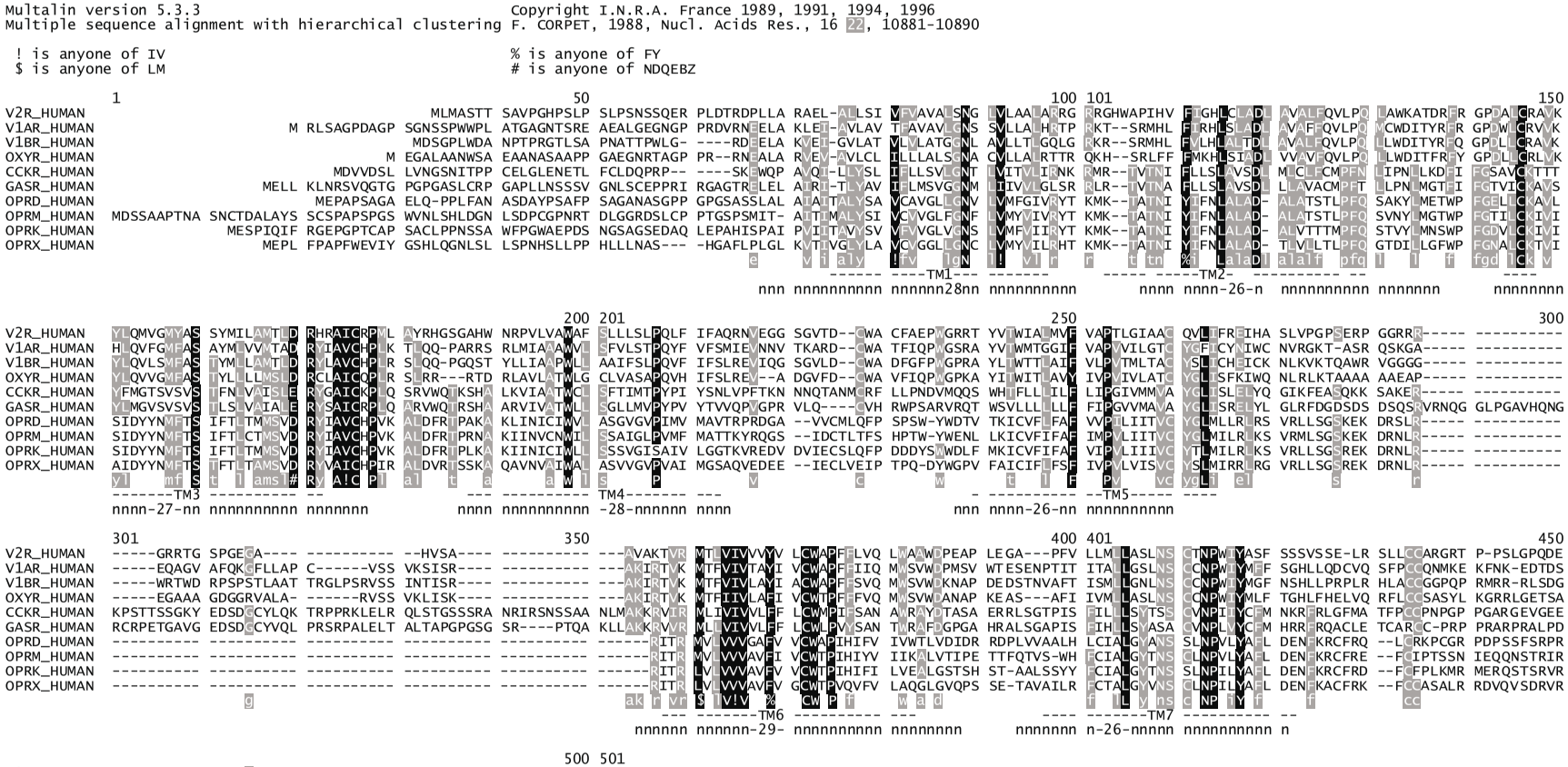
The AutoDock v. 2.4 program (Morris *et al.*, 1996) was used for docking of up to 4000 sterol molecular configurations by/around the surface of a receptor. AutoDock enables the docking of a flexible ligand (i.e. a sterol in this work) to a stiff host (receptor model) within a space confined to a rectangular block of a properly chosen size, see Fig. 2. Only non-bonded (van der Waals and electrostatic) interactions are taken into account in docking. For more details of our docking procedure(s) see (Giełdoń *et al.*, 2000). Due to a very rudimentary force field driving the docking, immediate configurations resulting from AutoDock are quite raw and indiscriminate. Therefore, we imposed selection criteria completely ignoring energy and accepting only those sterol configurations in which 75% of all atoms were located no further than 4 Å away from the receptor surface. A special "filter" program was written to this end.

Computations were done using an SGI Origin2000 8xR10000 and 4xR12000 computer in the Academic Computer Center in Gdańsk TASK and/or on a Sun UltraSpark10 in the Institute of Biochemistry, Johannes-Gutenberg-University in Mainz. The images for presentation were prepared using the MolMol program (Koradi *et al.*, 1996).

RESULTS

Conformational analysis of sterols

Recently, the effects of three groups of cholesterol analogues on high affinity binding of oxytocin and cholecystokinin by OTR and CCKRβ were described. The first group included modifica-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Multiple sequence alignment with hierarchical clustering F. CORPET, 1988, Nucl. Acids Res., 16 22, 10881-10890
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V2R_HUMAN
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OXYR_HUMAN
CCKR_HUMAN
GASR_HUMAN
OPRD_HUMAN
OPRM_HUMAN
OPRK_HUMAN
OPRX_HUMAN
TM1
TM2
TM3
TM4
TM5
TM6
TM7

tions in the linear tail, the second included modifications in the ABCD-ring frame, and the third one – combinations thereof, 24 sterols in total (Gimpl *et al.*, 1997). In this work, in addition to the two most stable conformational forms of cholesterol, we studied the strongest and the weakest OTR stimulators of the first group, *viz.* campesterol (24-methylcholesterol) and 25-hydroxycholesterol, respectively of the second group, *viz.* 7-dehydrocholesterol and 4-cholesten-3-one, respectively. In addition, we studied epicholesterol, a very week OTR stimulator from the second group in its two most stable conformations (having 3-OH in α, contrary to the β configuration in cholesterol). All sterols in their starting conformations are shown in Fig. 3.

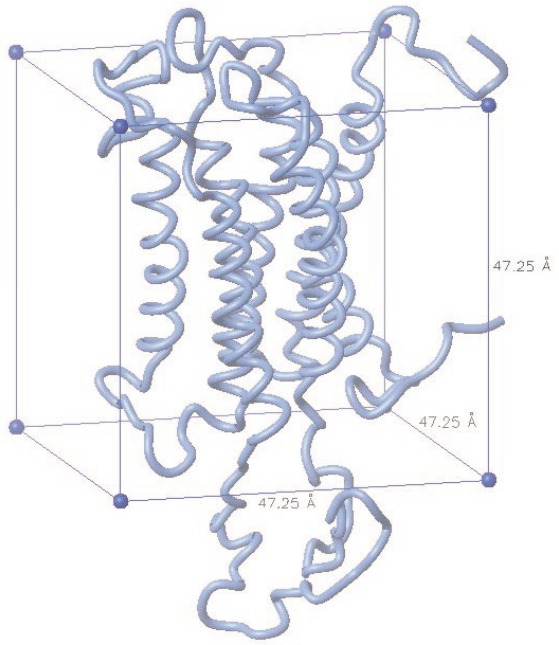

Figure 2. The size and the orientation of the docking imposed on the receptors for the sterol docking. AutoDock requires a pre-calculated *grid maps*, one for each atom type present in the ligand being docked.

This helps to make the docking calculations extremely fast. A grid map consists of a three-dimensional lattice of regularly spaced points, surrounding (either entirely or partly) and centered on some region of interest of the macromolecule under study (i.e. in our simulations OTR, CCKR and the mutant). Each point within the grid map stores the potential energy of a "probe" atom or functional group that is due to all the atoms in the macromolecule.

The conformational analysis has demonstrated that most of the compounds studied (except for 4-cholesten-3-one) prefer an ideal or only slightly perturbed (e.g. in 7-dehydrocholesterol) corrugated planar ABCD-ring system. The flexible hydrocarbon tail exhibits two types of conformational preferences: one, planar and typical of the solid-state structure of cholesterol (Shieh *et al.*, 1981), being simply an extension of the corrugated ABCD sheet in the common plane, and the second, having the tail bent at the C-20 carbon. Both structures have similar energies and they are still further distributed into more locally divergent subfamilies of conformations of comparative energies (differing at most by a few kcal/mol). The extra methyl C-24 in campesterol imposes a more distinct bent at C-20, accompanied with a stronger conformational diversity at the tail than that typical of cholesterol.

Cholesterol and epicholesterol were flexibly AutoDock-ed to the receptors, starting from both their representative planar and bent conformations, whereas campesterol, 25-hydroxycholesterol, 7-dehydrocholesterol and 4-cholesten-3-one were AutoDock-ed starting from their bent conformations only. This choice seemed justified because more OTR-docked cholesterol configurations were eventually sampled for the bent cholesterol than for the planar one, furthermore, a number of those arising from the starting planar cholesterol changed their conformations into bent ones on docking.

Oxytocin receptor

Using the box as defined in Fig. 2, 4000 configurations of receptor–sterol complexes were sampled for each sterol, which, submitted to filtering

Figure 1. Multiple sequence alignment for four human neurophyseal (oxytocin, V2, V1a and V1b), four opiate (μ, δ, κ and orphanin) and two (α and β) cholecystokinin receptors.

Putative TM helices are marked with the symbols --TM#-- and nnnnnnnnnn according to (Baldwin, 1993) and (Pogozheva *et al.*, 1998), respectively.

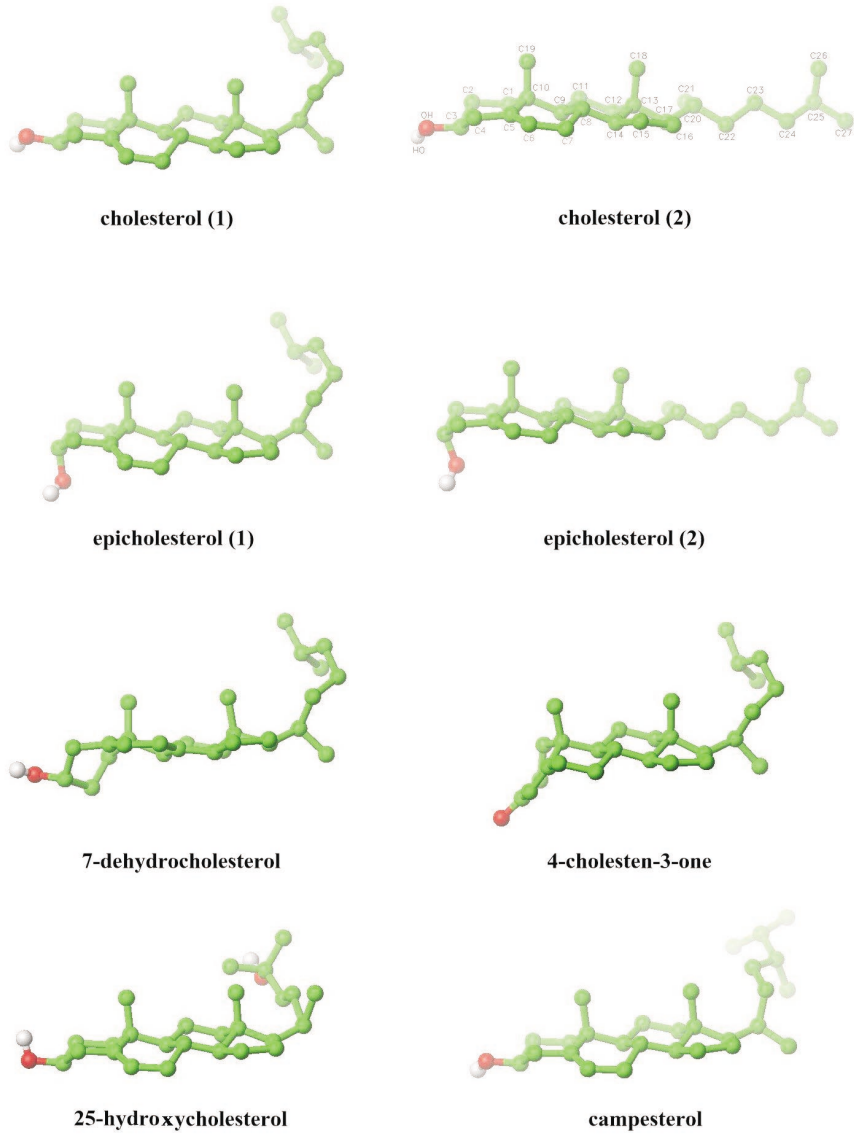

Figure 3. Starting conformations of sterols subject to current study.

Note that cholesterol and epicholesterol were considered in two conformations, termed planar and bent, see text.

(see Methods: 75% of atoms of the ligand no further than 4 Å away of the receptor surface), reduced to 10–90 configurations for OTR and the mutant and to 200–400 configurations for CCKRβ. The results are given in Fig. 4.

Each sterol/OTR set eventually produced 4–6 sterol clusters around the OTR surface, of which only one seemed to exhibit features common and unique to the activating sterols (including the bent cholesterol) while simultaneously orienting itself by the receptor lateral surface like a lipid, i.e. with its 3-hydroxyl group oriented towards the surface of the putative phospholipid bilayer and the remainder of the ABCD ring system plus the aliphatic tail progressing deeper into the bilayer, approximately parallel to the bilayer normal. This cluster centred around five hydrophobic residues at the extracellular half of the OTR surface, *viz.* P197, Y200 and W203 of transmembrane helix 5 (TM5) and M296 and W297 of TM6, see Fig. 5.

The remaining clusters were located at the cytosolic or extracellular bilayer surface and close to the cytosolic and extracellular domains of the OTR. The sterols were oriented randomly or

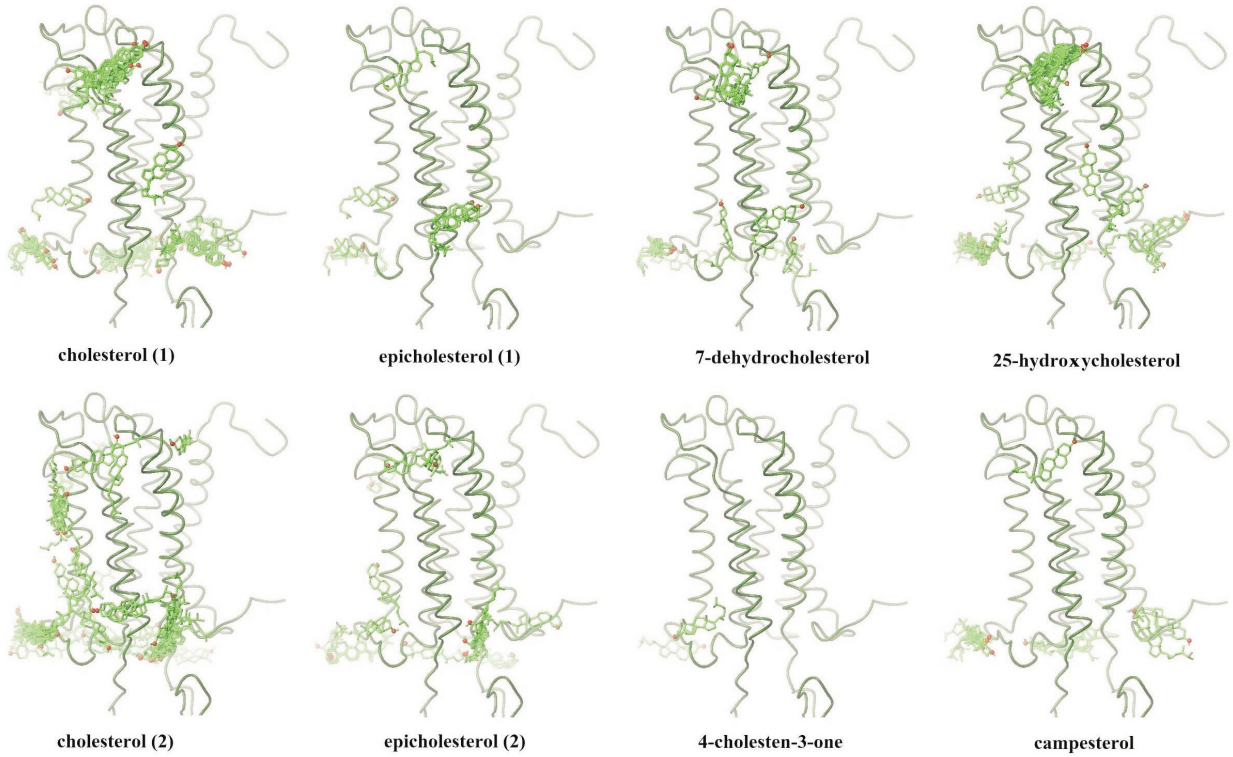

Figure 4. Distribution of sterols around the OTR model.

parallelly to the bilayer normal, hence not fitting our criterion of putative specific interaction with OTR. 25-Hydroxycholesterol, despite little active (Gimpl *et al.*, 1997), also exhibited a relatively abundant cluster in the TM5 and TM6 region. However, both 3-OH and 25-OH groups did not discriminate their positions, and also a considerable number of 25-hydroxycholesterol molecules substantially deviated from the bilayer normal direction, see Fig. 4.

Cholecystokinin receptor

The same set of sterols, docked under identical conditions by the surface of CCKR (Fig. 6) did not exhibit any sterol selectivity. Even though sizeable clusters result from each set of sterol docking, the clusters seem to be completely indiscriminate between active and inactive sterols and located at the lipid-water interface and at an extensive area near TM7 on the cytosolic side of the CCKR. Orientations of the docked sterol molecules are quite random.

The mutant

The mutant consisted of the OTR, in which the residues found as potentially sensitive to cholesterol (see above) were replaced with the residues occupying equivalent positions in the CCKR. Thus, the following four changes were incorporated into the OTR mutant: P197R, Y200W, W203L, M296T. Despite some sequence homologies (compare Fig. 1), in the corresponding area almost no sterol residues are located at the surface of the mutant, see Fig. 7. There is only one molecule of planar cholesterol, however, having its hydroxyl group not directed toward the water phase, and one molecule of bent cholesterol, oriented approximately perpendicular relative to the bilayer normal. Similarly behaves the single molecule of 7-dehydrocholesterol docked in this

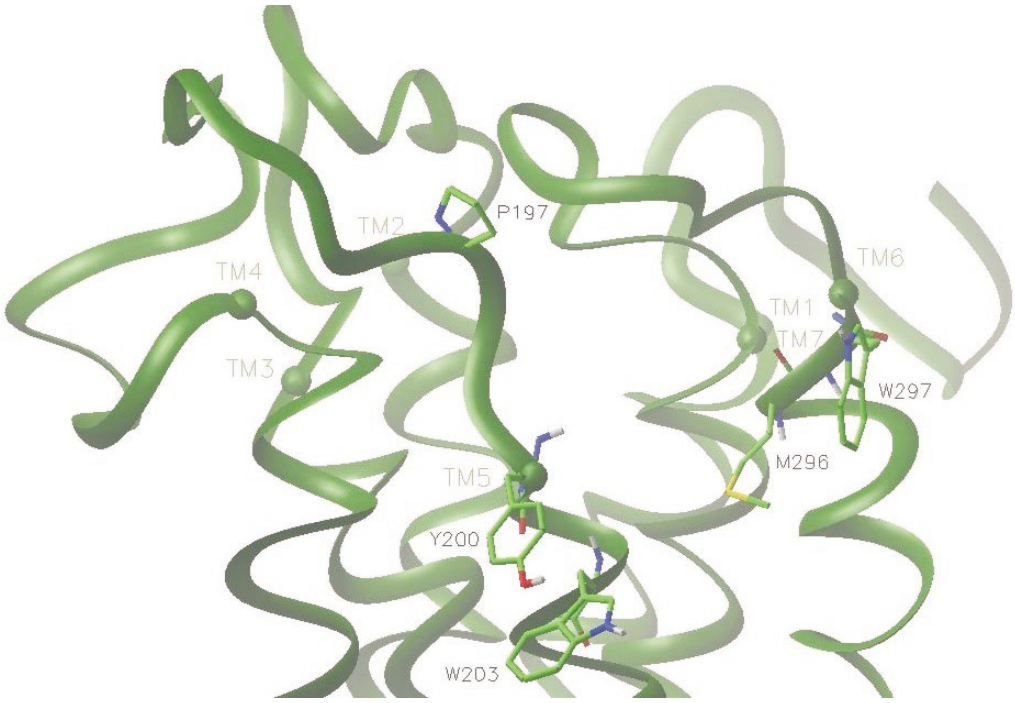

Figure 5. A putative interaction site between OTR and the activating sterols, see text.

area, see Fig. 7. 25-Hydroxycholesterol generates a cluster of indiscriminate configurations in this area, similarly as with OTR, see above.

DISCUSSION

Our molecular modelling indicates an area on the OTR surface potentially important for specific OTR-membrane cholesterol interactions, immersed not too deeply from the extracellular phospholipid bilayer-water interface and involving P197, Y200, W203 from TM5 and M296 and W297 from TM6. These residues, having extensive, largely non-polar side chains grouped together, seem particularly good candidates for specific interactions with the lipidic environment of the membrane bilayer. The positions of the docked cholesterol could be easily achieved by membrane cholesterols without perturbation of its membrane orientation (Brzustowicz *et al.*, 1999).

It is thought that structural properties of sterols are a prerequisite for their membrane function(s). In addition to the already mentioned β-3-hydroxyl group, the other properties are a planar steroid core and an aliphatic tail (Bloch, 1983). Our simulations indicate that epicholesterol, having an α-3-hydroxyl group, does not bind to OTR like cholesterol. Regarding the steroid core, both 7-dehydrocholesterol and 4-cholesten-3-one have a perturbed corrugated planarity of their ABCD-ring systems in the B and A rings, respectively, see Fig. 2, yet the former is active and the latter is not (Gimpl *et al.*, 1997). Hence, it appears that a minor deviation of the ABCD-ring system from planarity could be tolerated in regard to the OTR–sterol interactions, provided the β-3-hydroxyl is retained. It has been shown that a high-affinity state of OTR may depend on specific conformations of cholesterol other than that responsible for structural modification of the phospholipid membrane (Gimpl *et al.*, 1997). Regarding the tail, molecular dynamics of cholesterol in the dipalmitoylphosphatidylcholine (DPPC) bilayer (Tu *et al.*, 1998) have demonstrated that, apart from the most favoured cholesterol conformations with the extended tail, there are also three reasonably populated conformations having *gauche* bonds at C23–C24, C22–C23 and C20–C22, in the ratio 0.55 : 0.12 : 0.11 : 0.12, in a reasonable agreement (0.72 : 0.08 : 0.08 : 0.12, respectively) with the crystal-structure analysis of cholesterol (Rohrer *et al.*, 1980).

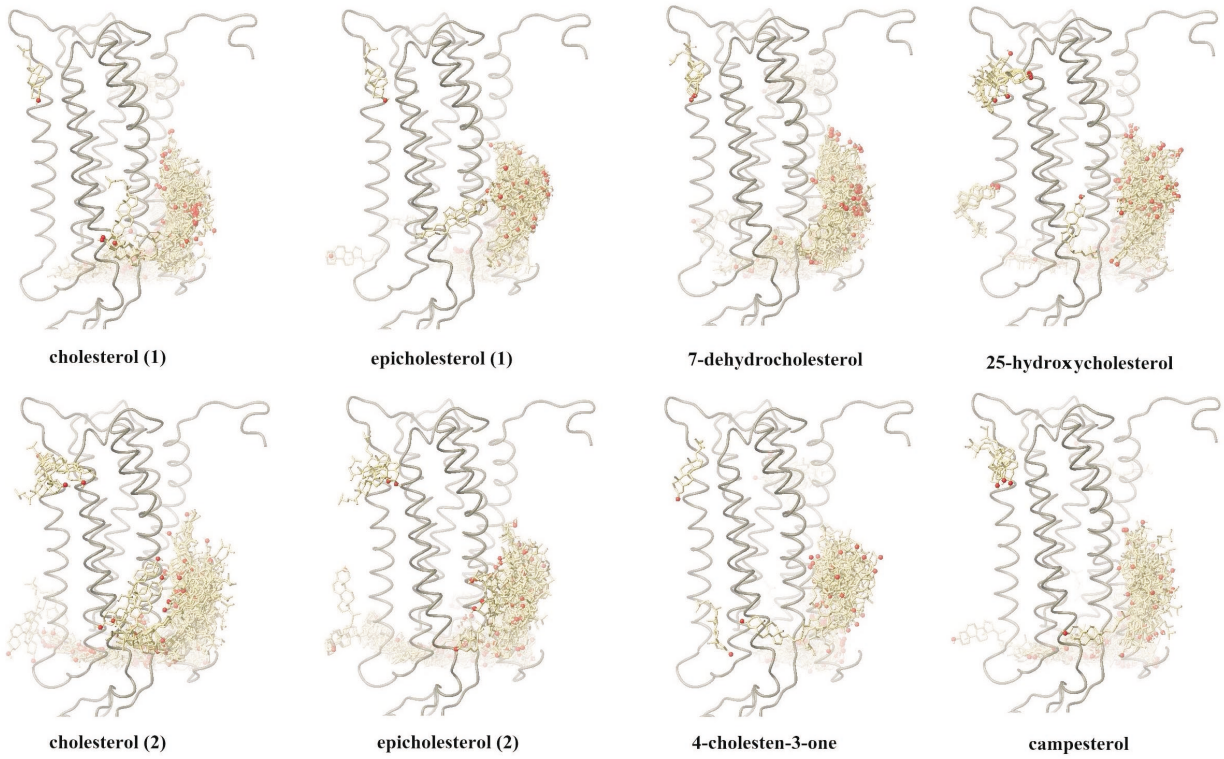

Figure 6. Distribution of sterols around the CKKR model.

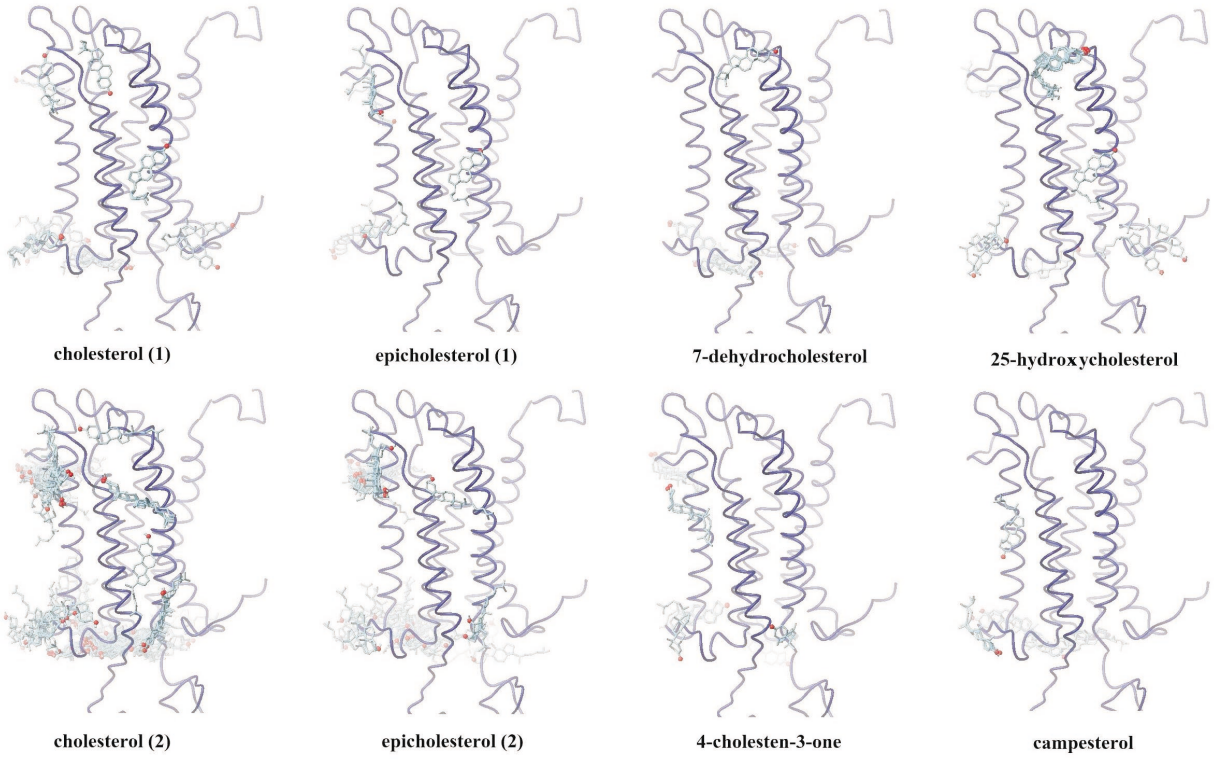

Figure 7. Distribution of sterols around the mutant receptor model.

Finally, our simulations indicate no sterol specifity towards CCKRβ as the docking results are similar for all of the investigated sterols. The location of the resulting cluster near the cytosolic domain and the random orientation of the docked molecules do not indicate any specific interaction.

In summary, the present study seems to be promising in most of its respects, confirming former experimental studies (Klein *et al.*, 1995; Gimpl *et al.*, 1997) by molecular modelling. Our theoretical predictions coupled with mutation-affinity studies may prove fruitful in further investigations of biochemical mechanisms pertinent to GPCR activation *via* membrane cholesterol.

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