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Review

Regulation of receptor function by cholesterol

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Abstract. Cholesterol influences many of the biophysical properties of membranes and is nonrandomly distributed between cellular organelles, subdomains of membranes, and leaflets of the membrane bilayer. In combination with the high dynamics of cholesterol distribution, this offers many possibilities for regulation of membrane-embedded receptors. Depending on the receptor, cholesterol can have a strong influence on the affinity state, on the binding capacity, and on signal transduction. Most important, cholesterol may stabilize receptors in defined conformations related to their biological functions. This may occur by direct molecular interaction between cholesterol and receptors. In this review, we discuss the functional dependence of the nicotinic acetylcholine receptor as well as different G protein-coupled receptors on the presence of cholesterol.

Key words. Rhodopsin; oxytocin; cholecystokinin; galanin; phospholipid; membrane fluidity; calcium measurement.

Cholesterol

Cholesterol, generally known as a major risk factor of arteriosclerosis, is an essential lipid of many eukaryotic organisms. It is the precursor of steroid hormones such as gestagens and mineralocorticoids and plays an important role in the structure of biological membranes. In eukaryotic cells, about 65–90% of cellular free (i.e., unesterified) cholesterol may reside in the plasma membrane [1] where its average concentration is about 200–300 μg/mg protein and the molar ratio of cholesterol to phospholipids may be as high as 1:1 (depending on the cell type) [2].

The chemical structure of cholesterol is shown in figure 1. Cholesterol contains a bulky steroid nucleus with a 3β-hydroxyl group at one end and a flexible hydrocarbon tail at the other. As illustrated in figure 1, cholesterol inserts into lipid bilayers with its long axis perpendicular to the plane of the membrane and may partly form tail-to-tail dimers [3]. The polar hydroxyl group of cholesterol might interact with a carbonyl oxygen atom of a phospholipid head group, whereas the hydrocarbon tail of cholesterol is located in the nonpolar core of the bilayer.

Which physical parameters of a biological membrane are influenced by cholesterol? First, cholesterol modulates the fluidity of biological membranes. It prevents the crystallization of fatty acyl chains by inserting between them, and at high concentration it abolishes phase transitions of bilayers. Cholesterol prevents large motions of fatty acyl chains, thereby making membranes less fluid. This ordering effect is accompanied by an increase in *staggered* conformations of the upper portion of the lipid hydrocarbon chains. This leads to an increase in bilayer thickness and to a decrease in membrane permeability. As mainly deduced from monolayer studies, cholesterol has a condensing effect, i.e., it decreases the surface area per molecule occupied by saturated and monounsaturated phospholipids. But

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cholesterol has only modest effects on the lateral diffusion of phospholipids. For more detailed information we recommend the review by Yeagle [2].

Biological membranes

Over the years, more detailed models of biological membranes have been developed. In 1972, Singer and Nicolson [4] proposed that the lipid bilayer could be a solvent for integral membrane proteins. According to their 'fluid mosaic model,' membrane proteins can diffuse laterally in a 'lake' of lipids but are unable to switch from one side of the membrane to the other, thereby maintaining the asymmetry of the bilayer. In the last few years, experiments have been performed to refine this model. Lipids are now known to be asymmetrically distributed between the two sheets of the bilayer (reviewed in refs [5, 6]). Controversy exists as to whether cholesterol is enriched in one of the two halves of the bilayer. Cholesterol can rapidly flip-flop between the leaflets of the lipid bilayer even in lipid vesicles (half time of less than 1 min at 37 °C [7]). In human platelet plasma membranes, cholesterol is reported to be enriched in the outer leaflet of the bilayer [8]. In contrast, Schroeder and colleagues [9] consistently found cholesterol to be concentrated in the inner leaflet in a variety of cell types.

There are lateral microdomains of lipids and proteins in the plane of the membrane which can differ in phase, in charge, in glycolipid, sphingolipid or cholesterol content [for a review see ref. 10]. In the case of cholesterol, three domains have been proposed: a cholesterol-poor fast-exchanging fluid phase where monomeric cholesterol is

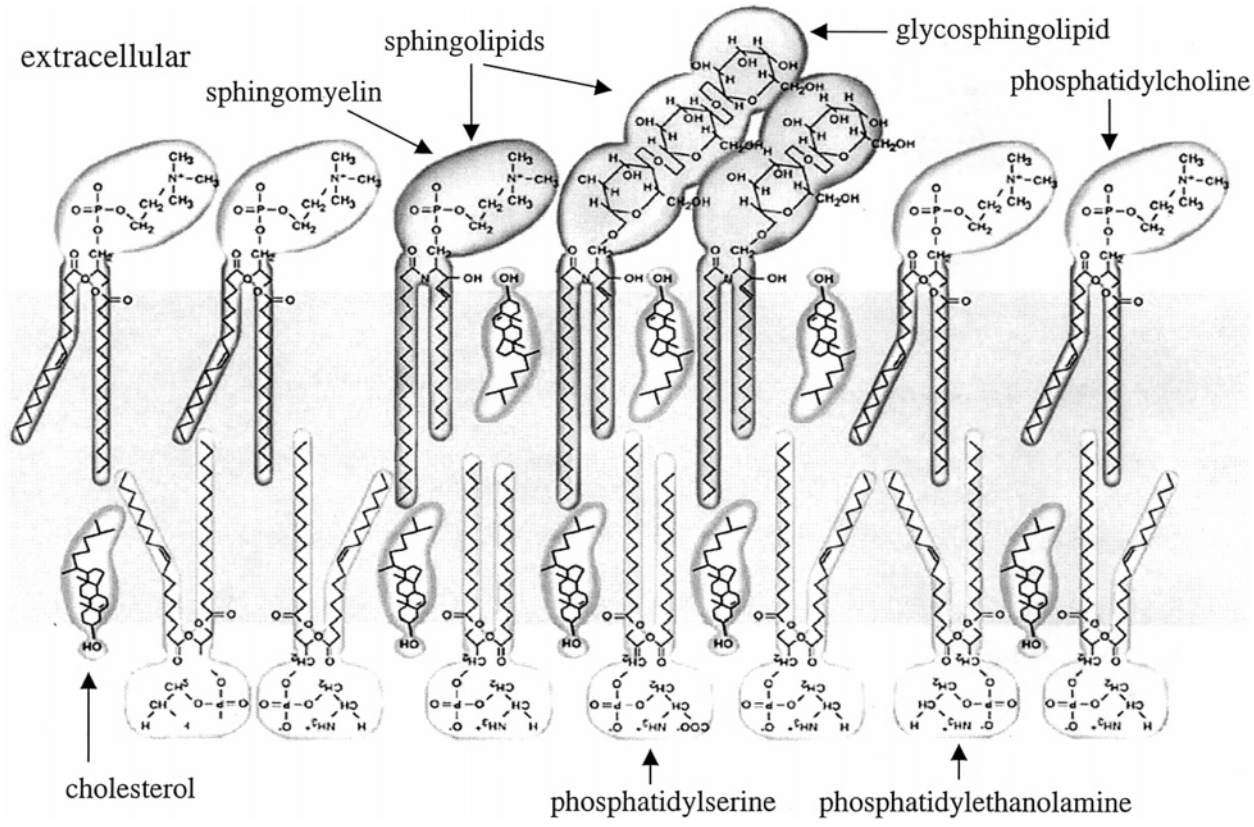

Figure 1. Hypothetical model of the lipid organization in raft mircrodomains [13]. In the plasma membrane, choline-containing lipids [phosphatidylcholine (PC) and sphingomyelin (SM)] are enriched in the extracellular leaflet of the bilayer, whereas amine-containing lipids (phosphatidylethanolamine and phosphatidylserine) are preferentially found in the inner leaflet [5]. Sphingolipids (SM and glycosphingolipids) and intercalating cholesterol form a tightly packed microdomain in a more fluid PC-rich environment. Cholesterol might fill the voids that tend to form underneath large head groups of the sphingolipids between the saturated hydrocarbon chains of the ceramide moieties. In addition, cholesterol could fill spaces which might originate between long ceramide fatty acyl chains that could partially interdigitate into the cytoplasmic lipid leaflet [95]. This model predicts a central role for cholesterol in the organization of membrane rafts. Reprinted by permission from *Nature* [12] Copyright (1997) Macmillan Magazines Ltd. and from Curr. Opin. Cell Biol. [13] Copyright (1997) Elsevier Science.

randomly distributed in the bilayer and migrates rapidly from one leaflet to the other, a less dynamic but still exchangeable cholesterol-rich fluid phase where cholesterol is present in both monolayers and alternates with phospholipid molecules, and a very slowly or nonexchangeable nearly pure cholesterol domain. With respect to the interaction of membrane-bound receptors and cholesterol, glycosphingolipid- and cholesterol-enriched microdomains or 'rafts' are of special interest (fig. 1). The 'raft' concept was introduced by Simons and van Meer [11]. Rafts are small and very dynamic membrane patches and have been implicated in many important cellular processes, such as polarized sorting of apical membrane proteins in epithelial cells and signal transduction [for reviews see refs 12–14].

Caveolae are small invaginations of the plasma membrane and are considered to be a specialized form of raft that contain the cholesterol-binding structural protein caveolin [15]. Several membrane receptors such as platelet-derived growth factor receptor and the m_2 muscarinic acetylcholine receptor, receptors for epidermal growth factor, adrenalin, bradykinin, endothelin, cholecystokinin, and oxytocin are enriched in caveolae or caveolae-like domains. In caveolae, receptors seem to be functionally connected to molecules of the signaling cascades, e.g., G protein α subunits, protein kinase Cα, mitogen-activated protein kinase, adenylyl cyclase and phosphoinositides [reviewed in ref. 16]. Caveolin interacts with some proteins involved in signal transduction such as G protein α subunits and regulates their activity [for a review see ref. 17]. Couet et al. [18] identified a caveolin-binding motif which is present in many caveolae-associated proteins, e.g., G protein α subunits, protein kinase Cα, mitogen-activated protein kinase, and receptors such as epidermal growth factor receptor, platelet-derived growth factor receptor, insulin receptor, and some G protein-coupled receptors (GPCRs), e.g., β-adrenergic receptor, endothelin receptor, and muscarinic acetylcholine receptor. Whether all these molecules actually interact with caveolin is not yet known, but caveolin could possibly influence the activity of several proteins of the signal transduction cascades.

Caveolae may also play a role in calcium signal generation. Isshiki et al. [19] recently demonstrated that in endothelial cells, calcium waves upon intracellular calcium release originate from caveolae-rich cell edges. Caveolin and caveolae also play a role in intracellular cholesterol transport [20, 21] and cholesterol efflux to extracellular carriers [22]. These findings indicate that dynamic cholesterol-dependent regulation of specific signal transduction pathways could take place within caveolae.

Taken together, cholesterol influences many of the biophysical properties of membranes and is nonrandomly distributed between cellular organelles, subdomains of membranes, and leaflets of the membrane bilayer. Moreover, the cholesterol distribution in membranes is highly dynamic [23]. This offers many possibilities for the influence of cholesterol on membrane-embedded receptors which we will discuss in this review.

Modes of receptor regulation by cholesterol

In principle, cholesterol can modulate membrane receptor function by the following two distinct mechanisms. It can either bind directly to the protein thereby altering, for example, the conformation of the protein, or it can influence the receptor indirectly by changing the biophysical properties of the membrane lipid bilayer. Below, we elucidate these two mechanisms on membrane-embedded receptors such as the nicotinic acetylcholine receptor (AChR) and GPCRs.

Nicotinic acetylcholine receptor

The AChR is a ligand-gated ion channel located, for example, in the membrane of muscle cells facing the axon terminal of a motoneuron. Binding of the neurotransmitter acetylcholine opens the channel leading to a large inward current of Na^+ and a smaller outward current of K^+, which results in a depolarization of the muscle cell. Since the acetylcholine receptor channel is very densely packed in membranes of the electric organ of *Torpedo*, an electric fish, it can be easily purified and studied in detail. It is a multimeric protein complex of five subunits (in the case of *Torpedo*: $\alpha_2\beta\gamma\delta$) that are symmetrically positioned around the channel pore. Each subunit contains four membrane-spanning segments M1–M4. The M2 domains from each subunit line the wall of the ion channel along the central axis of the pentamer [24]. Three rings of negatively charged residues have been suggested to modulate channel conductance and selectivity [25]. The M4 domain is the most hydrophobic and the least conserved and is believed to be in contact with the surrounding lipids [26, 27].

The interaction of the AChR with the surrounding lipids of the membrane has been studied for more than 20 years. In the late 1970s, delipidation of the AChR was found to modify the affinity of the AChR for agonists [28, 29]. In addition, solubilization of the AChR in detergents without adequate lipids irreversibly stabilized the AChR in a low-affinity state [30]. This low-affinity state is different from another low-affinity state which is capable of undergoing state transitions to a high-affinity state in the process of desensitization. Desensitization occurs during exposure of the AChR to certain cholinergic ligands and leads to blocking of the

channel opening. These concentrations and time-dependent state transitions are observed in the membrane-bound AChR and are normally lost upon solubilization in anionic or nonionic detergents, unless the latter are supplemented with exogenous lipids [31]. Therefore, these agonist-induced state transitions were initially regarded as a useful tool for determining the lipid requirements of the AChR. Cholesterol was thought to be absolutely necessary for the affinity transitions. Criado et al. [30] found that phosphoglycerides alone were not sufficient for preservation of agonist-induced state transitions independent of their polar heads and/or acyl chain composition . However, Fernandez-Ballester et al. [32] found that desensitization is just a function of prolonged exposure to the cholinergic agonists and does not depend on the presence of cholesterol or any other specific lipid in the reconstituted bilayer.

Another biologically relevant activity of the AChR is the ion-gating activity which is very sensitive to the lipid environment. Reconstitution in the absence of neutral lipids (= purified asolectin phospholipids) results in a pronounced decrease in the ability of the AChR to allow cation translocation in response to binding of cholinergic agonists [32]. In general, cholesterol is required [33], whereas unsaturated free fatty acids inhibit ion-gating activity [34]. It is interesting to note that the structure-activity requirements for cholesterol are not very stringent—even neutral lipids that are very dissimilar to cholesterol, e.g., vitamin D_3 can support the ion-gating activity [35]. Newer studies of Fernandez-Ballester et al. [32] revealed that restoration of AChR structure and function by cholesterol does not occur when the AChR is reconstituted in vesicles made from purified egg phosphatidylcholine (PC). This confirms studies which suggest that phospholipids other than PC may be required for cholesterol to exert its modulatory effects. Acidic phospholipids such as phosphatidic acid, cardiolipin, or others are necessary along with cholesterol to allow optimal control of AChR cation gating [33, 35–38]. Those phospholipids could also be required to enable cholesterol to act as a structural modulator of the AChR. Phosphatidic acid interacts strongly with the AChR and itself serves as a protein structural effector [39].

What is the nature of the interaction between cholesterol and the AChR? In 1994, Sunshine and McNamee [40] and Fernandez-Ballester et al. [32] showed that the action of the ion channel does not correlate with modulations of membrane fluidity. However, Sunshine and McNamee [40] discussed that an optimal fluidity of the lipids *directly* surrounding the AChR could be necessary for the function of the receptor. Experiments with spin-labeled steroid probes [41, 42], infrared spectroscopy [43], or calorimetry [44] support the hypothesis of a direct interaction of cholesterol with the receptor. In addition, all four different subunits of the AChR in membrane vesicles have been labeled with [^{3}H]cholesteryl diazoacetate, a cholesterol derivative where the 3-hydroxyl group is replaced by a photoreactive group [45] and 3α-(4-azido-3-[^{125}I]-iodosalicylic)-cholest-5-ene ([^{125}I]azido-cholesterol) [46]. All subunits of the AChR have also been labeled with photoreactive phospholipid probes [26]. These experiments demonstrate that the AChR interacts directly both with cholesterol and phospholipids.

Numerous groups have explored the putative cholesterol-binding sites of the AChR. Interestingly, high percentages of cholesterol are found in electroplax [47] and in postsynaptic membranes [48]. In 1978, Marsh and Barrantes [49] reported a motionally restricted shell of lipids (annulus) encircling the AChR in native membranes from *Torpedo marmorata*. Annular lipids exchange with neighboring lipids much more slowly (10^4–10^6 s) than bulk lipid (10^7 s) [for review on lipid domains see ref. 10]. On the basis of experiments on the interaction of lipids and the (Ca^{2+}-Mg^{2+})-ATPase, the group of Lee [50] postulated that the binding of hydrophobic molecules occurs at three distinct classes of site: bulk lipids, the lipid-protein interface (annular sites), and sites on the protein from which phospholipids are excluded (nonannular sites). The ion-gating activity of the AChR is probably not influenced by bulk lipids, because the activity does not correlate with the fluidity of the membrane (see above). In 1988, Jones and McNamee [51] published quenching studies with an AChR which had been purified and reconstituted in membranes with or without brominated lipids. The AChR of *T. californica* contains 51 tryptophans and therefore exhibits an intrinsic fluorescence. This fluorescence is partially quenched upon incorporation of brominated lipids into the membrane, and the interaction of lipids with the AChR can be quantified. Interestingly, while cholesterol could not displace brominated phospholipids from the AChR, bromocholesterol quenched the fluorescence of the AChR reconstituted in dioleoylphosphatidylcholine vesicles. In addition, reconstitution of dibromocholesterol with vesicles containing brominated PC and purified AChR led to a further reduction in fluorescence intensity. The authors concluded that cholesterol interacts directly with the AChR at the nonannular sites from which phospholipids may be excluded but binds less strongly to the annular sites. In contrast, fatty acids appear to bind to both annular and nonannular sites. Jones and McNamee proposed five to ten nonannular binding sites located in the interstices of the five receptor subunits.

Five years later, the group of McNamee confirmed the hypothesis of cholesterol-binding sites which are not accessible to phospholipids. They selectively labeled the γ subunit of the *T. californica* AChR at specific cysteine residues of the M4 helix with pyrene and measured the

quenching of the fluorescence of the cysteine-bound pyrene after addition of brominated cholesterol hemisuccinate and PC [52]. Cholesterol hemisuccinate retains the function and properties of cholesterol (although it lacks the 3β-OH) with respect to its interaction with both the membrane [53, 54] and AChR [30, 37]. Desensitization of the AChR in the presence of 1 mM carbamylcholine increased the accessibility to cholesterol by 20–25% whereas in the case of the unlabeled AChR (intrinsic fluorescence of Trp), the quenching by cholesterol was reduced. The authors concluded that the ligand-induced conformational changes are sensed at the protein-lipid interface, supporting a possible functional linkage between the membrane environment and ligand-mediated ion conductance changes. Given the possibility that the four transmembrane-spanning domains of each subunit of the AChR are α-helical, the cholesterol-binding domains are probably located at intersubunit sites and/or interhelical sites (fig. 2). In that case, the pyrene-labeled Cys451 and Trp453 of the M4 helix of the γ subunit of *Torpedo* AChR would be on opposite sides of the helix. This could explain the complementary changes observed in quenching of the fluorescence of the pyrene-labeled and unlabeled AChR upon desensitization [52].

The findings of a possible rearrangement of transmembrane portions of the AChR during prolonged exposure to cholinergic agonists are supported by Fernandez et al. [55] who labeled the AChR with *p*-azidophenacyl 3α-hydroxy-5β-cholan-24-ate (APL). The cholinergic agonist carbamylcholine but not α-bungarotoxin or the competitive antagonist (+)-tubocurarine partly prevented AChR photolabeling by APL. Carbamylcholine altered the distribution of the label among the AChR subunits and resulted in relatively greater photolabeling of the α subunit. On the other hand, Corbin et al. [46] reported that labeling patterns of the AChR with [^{125}I]azidocholesterol were the same in the presence or absence of carbamylcholine. They concluded that structural transitions in the receptor upon addition of agonist do not significantly change the surface area of the receptor exposed to the steroid. These authors propose that cholesterol might act as a sort of 'molecular grease' that facilitates subtle structural movements of the transmembrane segments during conformational transitions.

Experiments with spin-labeled lipids (phospholipids, androstane, and stearic acid) in native receptor-rich membranes of *T. californica* were performed by Dreger et al. [56]. After removal of the extramembrane portions of the receptor by proteinase K, binding sites for androstane and stearic acid but not for phospholipids were lost. This confirms the existence of topologically distinct lipid-binding sites for phospholipids (annular binding sites) and for cholesterol (nonannular binding sites). In contrast to the results of McNamee's group, Dreger et al. could not detect significant changes in lipid-protein interactions upon desensitization of the AChR. They concluded that the overall structure of the transmembrane domain could be rigid, and small changes could be sufficient to define different functional states of the receptor. In addition, Dreger et al. found that tyrosine phosphorylation does not affect the selectivity of the interaction of the AChR with different lipid species. Tyrosine phosphorylation regulates in vitro the rate of receptor desensitization [57] and induces anchoring of the AChR of neuromuscular junctions to the cytoskeleton [58]. According to former Fourier-transform infrared (FTIR) studies of Dreger et al., tyrosine phosphorylation leads to no detectable changes in the overall secondary structure of the receptor, albeit the authors did not exclude local conformational changes in the cytoplasmic region of the protein.

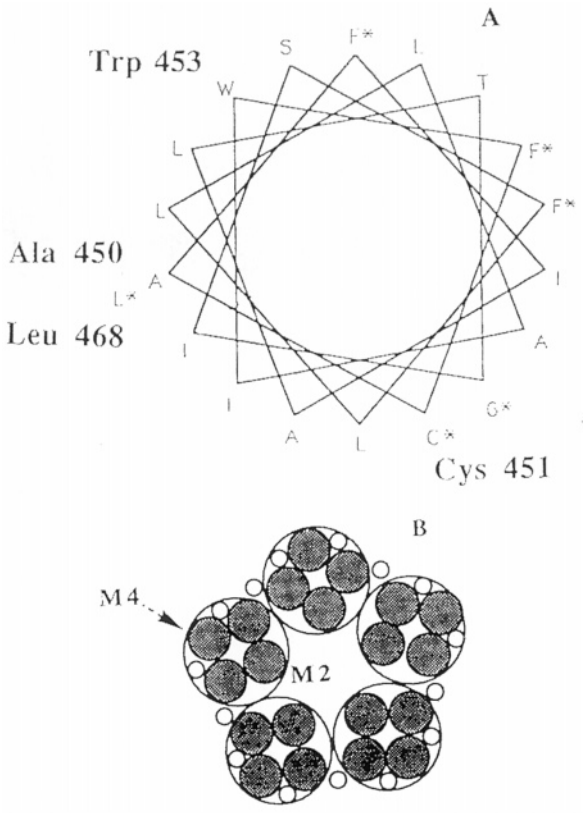

Figure 2. (*A*) Helical wheel plot of the putative M4 helix of the γ subunit of the *Torpedo* AChR, from Ala 450 to Leu 468, represented as single-letter amino acid codes. Conserved residues are shown with an asterisk. (*B*) Model of the AChR subunits (large circles) showing the four transmembrane domains (shaded medium circles) and the possible cholesterol-binding sites (small open circles). The domains believed to face the ion channel and the lipid milieu, M2 and M4, respectively, are indicated. Reprinted with permission from Narayanaswami and McNamee [52]. Copyright (1993) American Chemical Society.

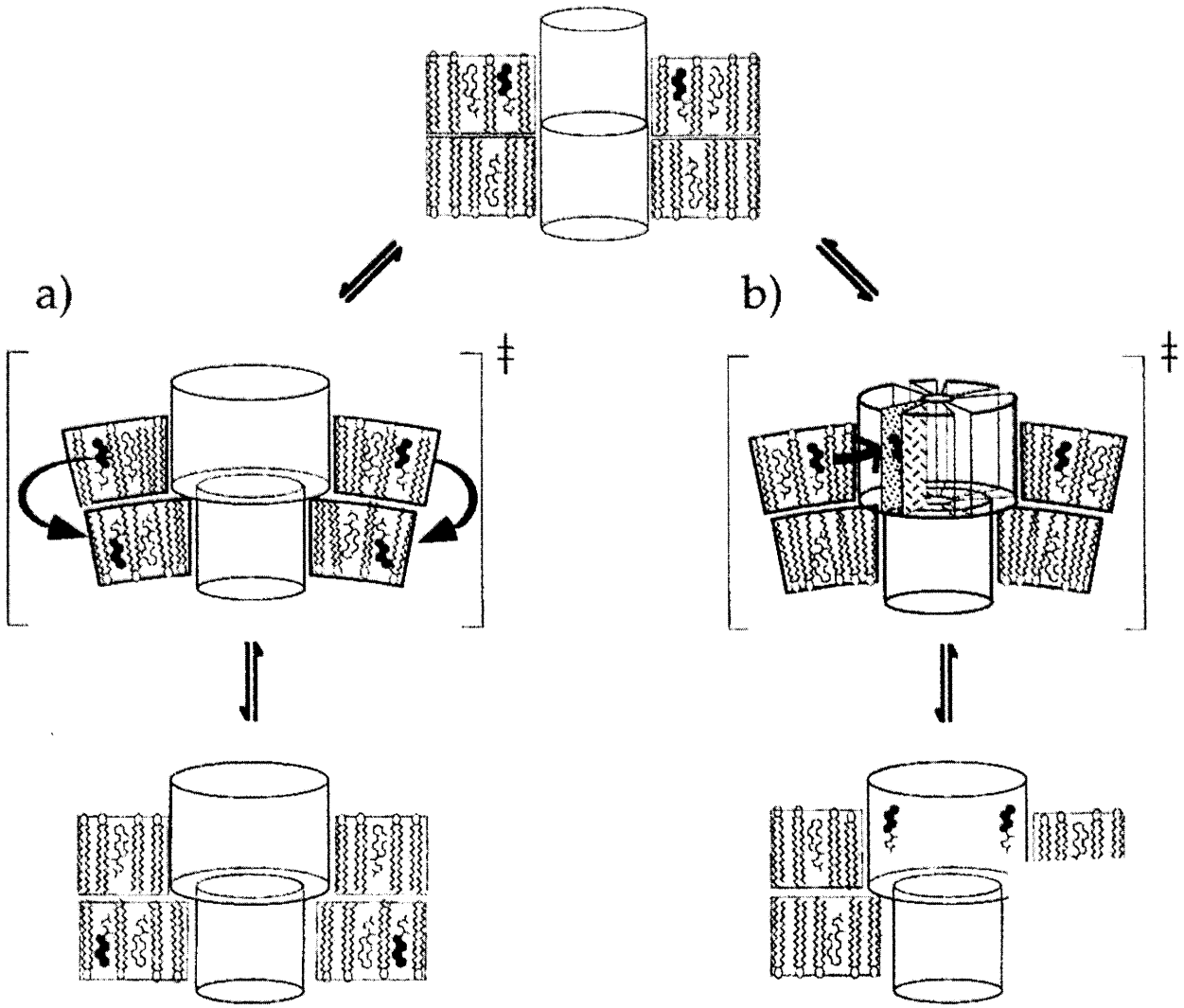

Figure 3. Models in which conformation-induced bending strain in the lipid bilayer is relieved by cholesterol [60]. This class of model assumes that activation of the AChR channel involves a conformational change in which the cross-sectional area of the channel increases in a nonuniform fashion. This results in a net asymmetric expansion in the two leaflets of the membrane's bilayer, increasing the mechanical strain of the bilayer. Relief of the mechanical strain, which can be achieved via two mechanisms, allows the conformation change to proceed and the channels to open. In both models, the receptor is initially in the resting state with the necessary amount of cholesterol in the bilayer. Upon activation, the induced bilayer stress is relieved in model (*a*) by cholesterol molecules rapidly flipping from the upper to the lower leaflet of the bilayer. In model (*b*), cholesterol-binding sites on the receptor became exposed during the conformation change. The induced bilayer stress is then relieved by cholesterol diffusing laterally to bind to the exposed sites on the receptor, stabilizing the open state. The cholesterol-binding sites would have to rapidly equilibrate with cholesterol, thus be readily accessible from the lipid-protein interface. For simplicity, only two bound cholesterol molecules are shown, but one within each subunit interface is more likely. Reprinted from Addona et al. [60]. Copyright (1998), with permission from Elsevier Science.

In addition to nonannular sites, there exist approximately 45 annular sites per AChR pentamer with a 20-fold lower affinity for cholesterol [51]. A minimum number of about 45 lipids per receptor is required to prevent irreversible loss of ion channel gating functions in native and reconstituted AChR [59].

Recent work from Addona et al. [60] dealt further with the hypothesis that cholesterol could bind to non-annular sites within the AChR itself. They synthesized a cholesterol-containing phospholipid (1-oleoyl-2-(cholesteryl hemisuccinyl)phosphatidylcholine) which resides at the lipid-protein interface. This 'tethered'-cholesterol analogue was able to restore the AChR activity in cholesterol-free 1,2-dioleoyl-*sn*-glycero-3-phosphate/1,2-dioleoyl-*sn*-glycero-3-phosphocholine membranes ruling out cholesterol-binding sites deeply

buried within the AChR. The authors concluded that cholesterol binds to sites of the AChR which are in contact with the lipid bilayer. Addona et al. suggested a new classification of nonannular binding sites into 'interstitial' sites, i.e., sites that are buried within the receptor, and 'periannular' binding sites. According to Addona et al., 'periannular' binding sites are located within crevices on the surface of the protein that are open to the lipid bilayer and that are distinct from annular binding sites.

Besides the discussion whether cholesterol binds to nonannular binding sites or to binding sites at the lipid-protein interface, there remains one question: what is the actual mechanism by which cholesterol maintains the function of the AChR? Addona et al. [60] proposed that cholesterol relieves conformation-induced bending strain which might occur upon ligand binding to the receptor (fig. 3). In their model, activation of the channel leads to an uneven increase in the cross-sectional area of the channel, i.e., the diameter of the receptor in one half of the bilayer increases more than in the other half of the bilayer. This results in mechanical strain of the bilayer which could be relieved by two possible mechanisms: (i) cholesterol could flip rapidly into the concave half of the bilayer, or (ii) the conformational change could lead to an exposure of cholesterol-binding sites on the receptor. Lateral diffusion of cholesterol to these sites could stabilize the open state of the receptor. Since Addona et al. found that charged cholesterol analogues which are unable to flip-flop rapidly between the two halves of the lipid bilayer support channel activation effectively, the first possibility can be ruled out. The second possibility implies that these binding sites for cholesterol are asymmetrical with respect to the bilayer and that a rapid exchange with the bilayer occurs ('periannular' binding sites). However, this hypothesis needs to be tested in the future.

Another important point of view is that lipids influence the secondary structure of proteins. In 1987, Fong and McNamee [43] performed FTIR analyses with lipids that support ion channel activity of the AChR. These lipids increased the apparent α helix (cholesterol) and β sheet (phosphatidic acid, cholesterol) content of the receptor [32, 43]. As measured by FTIR spectroscopy, increasing cholesterol concentrations in the reconstituted matrix containing purified asolectin phospholipids progressively restored the different secondary structural components to percentages as high as in the samples reconstituted in whole asolectin [32]. On the other hand, using the same method, Méthot et al. [61] did not detect significant differences in receptor secondary structure in the absence of cholesterol.

In addition to possible influences on protein secondary structure, cholesterol might stabilize the receptor protein in the native membrane, as shown for rhodopsin, a GPCR which we will discuss below. The phenomenon of agonist-induced thermal stabilization of the AChR is strongly diminished when the receptor is reconstituted in the absence of neutral lipids but it is easily regained when cholesterol is present in the reconstituted matrix [32].

G protein-coupled receptors

GPCRs are integral membrane proteins with seven putative membrane-spanning domains, an extracellular N terminus, and an intracellular C terminus. To date, more than 1000 members of this superfamily have been cloned. They are found in a wide range of organisms from yeast to mammals and are involved in the transmission of extracellular signals to the interior of the cell. GPCRs respond to a wide and diverse range of agents including catecholamines, peptides, lipid analogues and stimuli such as light, taste, and odor. Upon binding of a ligand, the GPCR undergoes conformational changes which allow the coupling of a heterotrimeric G protein (guanyl nucleotide-binding protein) to an intracellular loop of the GPCR. This leads to an exchange of GDP bound to the α subunit of the G protein versus GTP. In turn, the G protein dissociates into its α and $\beta\gamma$ subunits, both of which can modulate certain enzyme activities or ion channels.

Rhodopsin

Rhodopsin, the photoreceptor of rods, is one of the best-known GPCRs. Upon absorption of light, rhodopsin activates the GTP-binding protein transducin by catalyzing the exchange of GTP for bound GDP. In turn, the α subunit of transducin dissociates from the $\beta\gamma$ subunits and activates the enzyme phosphodiesterase. This enzyme hydrolyzes cGMP which is bound to cGMP-gated cation-specific channels of the plasma membrane. The hydrolysis of cGMP leads to closure of these channels followed by hyperpolarization of the rod cell and thus to the formation of an electrical signal [for a review see ref. 62]. This signal is processed by other nerve cells within the retina and is subsequently transmitted to the brain. Light induces a series of conformational changes in rhodopsin, during the course of which, the conversion of the photointermediate metarhodopsin I to metarhodopsin II produces the activated form of the receptor which in turn triggers the signal cascade described above.

The equilibrium between these two conformations of rhodopsin is sensitive to the cholesterol concentration of the membrane; that is, increasing the membrane cholesterol level shifts the equilibrium towards metarhodopsin I [63–65]. Thus, the function of

rhodopsin is impaired by high cholesterol concentrations, i.e., upon stimulation by light, rhodopsin in cholesterol-rich membranes fails to activate phosphodiesterase via transducin [66].

In the case of rhodopsin, both direct and indirect mechanisms of cholesterol action have been reported. In reconstituted rhodopsin-phospholipid membranes, modulation of the equilibrium between metarhodopsin I and metarhodopsin II by cholesterol was explained by a reduction in the free volume available for molecular motion in the hydrophobic core of the bilayer [65]. Since metarhodopsin II formation is accompanied by volume expansion of rhodopsin [67], a reduction of the free volume of the bilayer by cholesterol could inhibit metarhodopsin II formation. In contrast, Albert et al. [68] investigated a possible direct interaction between cholesterol and rhodopsin in bovine rod disk membranes. Disks are closed, flattened membranous sacs in the outer segment of a retinal rod cell, which harbor rhodopsin. These disks are formed by evaginations of the plasma membrane on the base of the rod cell and are displaced towards the apical tip of the outer segment as additional disks are formed. Old disks are shed at the apical tip and phagocytosed by the overlying retinal pigment epithelium. Albert et al. inserted a fluorescent sterol, cholestatrienol, in the disk membranes by exchange from donor phospholipid vesicles. Since the fluorescence emission from protein tryptophans overlaps the absorption bands of cholestatrienol, a fluorescence energy transfer will occur from the protein tryptophans to cholestatrienol if the two molecules approach closely. Rhodopsin represents more than 90% of the protein in disks [69]. Thus, tryptophan fluorescence from the disk membrane would be dominated by rhodopsin tryptophans. In fact, Albert et al. observed an energy transfer between rhodopsin and cholestatrienol as indicated by the quenching of tryptophan fluorescence by cholestatrienol. In addition, the specificity of this interaction could be shown by the ability of cholesterol but not ergosterol to decrease the quenching.

How many cholesterol molecules interact with one molecule of rhodopsin? Albert et al. [68] found that 20% of the membrane sterol is relatively inaccessible to oxidation. The authors assumed that this inaccessibility to cholesterol oxidase could be due to an interaction of cholesterol with rhodopsin. They calculated that one cholesterol molecule would interact with one molecule of rhodopsin. Furthermore, spin label experiments defining the phospholipid annulus of rhodopsin in rod outer segment disk membranes led to the conclusion that one sterol was found at the lipid-protein interface [70].

What could be the physiological implication of a direct or indirect interaction of cholesterol and rhodopsin? Interestingly, membranes of rod outer segments are heterogenous with respect to their cholesterol content. The cholesterol concentration (moles cholesterol to moles phospholipid) varies between 30 mole percent (newly formed basal disk membranes) and 5 mole percent (oldest disk membranes) [71]. The cholesterol concentration of new disks corresponds to the cholesterol concentration of the plasma membrane from which these disks are formed. How is this conspicuous cholesterol gradient obtained? One explanation is that the disk membranes have a much higher ratio of phosphatidylethanolamine to phospatidylcholine (0.92) than the plasma membrane (0.16) [72]. Since phosphatidylethanolamine-rich membranes provide a thermodynamically unfavorable environment for cholesterol, cholesterol moves from the disk membrane to the plasma membrane [for an excellent review see ref. 73]. In contrast, no change in fatty acid composition or in phospholipid to protein ratio among disks at different locations in the rod outer segment has been observed [71]. In view of these observations, rhodopsin function might increase with increasing age of the disk. In addition, the high cholesterol content of the plasma membrane might protect rhodopsin from thermal denaturation [74], thereby stabilizing rhodopsin until it reaches its place of function.

The importance of lipid sorting in rods is underlined by the phenotype of the Royal College of Surgeons (RCS) strain of rats [75]. The rhodopsin content of the photoreceptors of these animals is normal. Nevertheless, the animals are blind due to abnormal growth of their photoreceptors and the accumulation of membrane debris. The reason for this is a lack of lipid sorting in retinal photoreceptor cells so that the phospholipid composition of disk membranes is similar to that of the plasma membrane [76]. Therefore, there exists no phosphatidylethanolamine/phosphatidylcholine gradient and no difference in the cholesterol content of disk membranes and plasma membrane. This disease emphasizes the role of lipid composition in the maintenance of receptor and cellular function.

Oxytocin receptor versus cholecystokinin receptor

Here we summarize our results concerning the interaction of cholesterol with two different GPCRs, the oxytocin and cholecystokinin receptors. Oxytocin is a neurohypophyseal nonapeptide. It stimulates contraction of uterine smooth muscle cells [77] and is clinically used to induce labor. Oxytocin also stimulates milk secretion in response to suckling by inducing contraction of myoepithelial cells in the mammary gland [78]. Furthermore, oxytocin plays an important role in reproduction biology by influencing sexual behavior and response, as well as the formation of social bonds [79]. The function of cholecystokinin will be described below.

In the early 1990s, Klein and Fahrenholz [80] described reconstitution experiments with the oxytocin receptor (OTR) of guinea pig uterus. These experiments demonstrated that cholesterol is a prerequisite for the high-affinity binding activity (K_d in the low nanomolar range) of the myometrial OTR after reconstitution. In further experiments, the cholesterol content of native myometrial plasma membranes was changed by methyl-β-cyclodextrin [81]. Cyclodextrins are water-soluble cyclic oligosaccharides consisting of six to eight glucose molecules. Cyclodextrins enhance the solubility of nonpolar substances such as cholesterol by incorporating them into their hydrophobic cavity. Since β-cyclodextrin selectively extracts cholesterol from the plasma membrane in preference to other membrane lipids [82], it can be used to deplete membranes of cholesterol. Vice versa, by means of steroid-cyclodextrin complexes, steroids can be incorporated in membranes [81]. Removal of cholesterol ($> 95\%$) from the membrane of guinea pig myometrium increased the dissociation constant for [^{3}H]oxytocin from $K_d = 1.5$ nM to $K_d = 131$ nM, therefore shifting the OTR from high to low affinity without changing the number of binding sites ($B_{max} = 1.7$ pmol/mg). Replenishment of cholesterol-depleted membranes with cholesterol by incubation with cholesterol-methyl-β-cyclodextrin complexes restored the high-affinity binding. Intermediate cholesterol levels lead to an equilibrium between the low- and the high-affinity binding site and not to one population of binding sites with intermediate affinity. Interestingly, addition of cholesterol-methyl-β-cyclodextrin complexes to detergent-solubilized OTR induced a saturation of the solubilized binding sites for oxytocin. This suggests a direct interaction between the OTR and cholesterol, since in the soluble fraction, effects are eliminated which may be caused by properties of the lipid bilayer such as membrane fluidity, thickness, or curvature.

In this context, the structural requirements of the cholesterol-OTR interaction are of special interest. Klein et al. described first experiments with cholesterol analogues concerning this important issue. In 1997, we extended these experiments, to provide detailed information not only on the structural features of cholesterol which are important for its interaction with the OTR but also on the mode of this interaction [83]. In particular, we compared the human OTR with the human cholecystokinin receptor subtype B (CCK_BR). In the central nervous system, the CCK_BR has been implicated in the pathogenesis of panic-anxiety attacks [84]. Furthermore, CCK induces analgesia [85] and decreases exploratory behavior [86]. We chose the CCK_BR because it should not be dependent on cholesterol since it can be solubilized in a fully active form by digitonin [87, 88], a detergent which leads to complete depletion of cholesterol in the solubilized fraction due to the formation of insoluble 1:1 complexes with cholesterol [89]. In addition, both receptors use hydrolysis of phosphoinositol phosphates in their signal transduction pathway and thus could be compared with the same assay system in *in vivo* experiments. In our study, we used human embryonic kidney (HEK) 293 cells stably transfected with the human oxytocin receptor (HEK-OTR cells) and the human cholescystokinin receptor type B (HEK-CCK_BR), respectively. In membranes of HEK-OTR cells, methyl-β-cyclodextrin-mediated cholesterol depletion (removal of about 85% of the initial cholesterol) shifted the OTR from a high- to low-affinity binding state without altering the total number of binding sites. In contrast, the binding affinity of the CCK_BR remained unchanged but the capacity of the [^{3}H]propionyl-CCK8-binding sites was reduced to 45% of initial values. Again, these effects were reversible upon reloading of the membranes with cholesterol.

Treatment of HEK-OTR and HEK-CCK_BR membranes with cholesterol oxidase which catalyzes the conversion of cholesterol to 4-cholestene-3-one led to a decrease in oxytocin binding, most probably because of the substitution of the 3-OH group by a keto function, whereas the ligand-binding function of the CCK_BR was completely unaffected. Treatment of the membranes with this enzyme did not alter the membrane fluidity. As expected, an increase in membrane fluidity was observed when the membranes had been treated with methyl-β-cyclodextrin. In a further approach, we employed the cholesterol-binding fluorochrome filipin which sequesters cholesterol into patches without causing chemical modifications to the sterol. We observed a dose-dependent decrease in oxytocin binding ($IC_{50} = 44$ μM), whereas the cholecystokinin binding remained unchanged. These results supported our hypothesis that the CCK_BR could be influenced by the physical properties of the membrane whereas the OTR could be affected by a direct interaction with cholesterol.

For structure-activity analysis, we substituted membrane cholesterol by different steroids in both membranes of HEK-OTR and HEK-CCK_BR cells using the 'cyclodextrin-method.' What are the structural features of an 'active' steroid, i.e., a steroid that stabilizes the OTR in a high-affinity state or that conserves the capacity of CCK_BR binding sites? The results for the OTR are summarized in figure 4. This figure illustrates the modifications of cholesterol which restored (colored in green) the high-affinity binding function of the OTR in cholesterol-depleted membranes. Modifications leading to inactive steroids are shown in red.

In contrast, when we analyzed the structure requirements of the CCK_BR, we found no complete inactive steroid. Three different hydroxycholesterols exhibited the lowest efficiencies: 22(R)-, 25-, and 19-hydroxy-

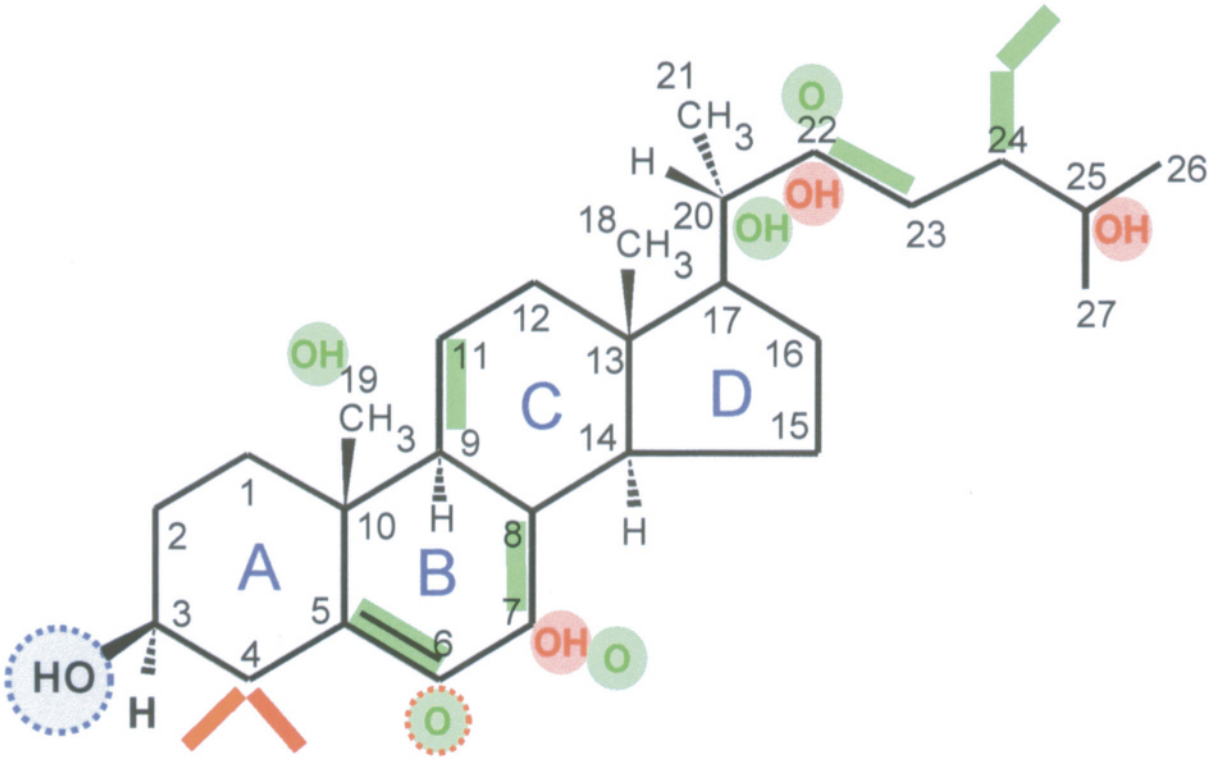

Figure 4. Structure-activity analysis of cholesterol with respect to the high-affinity binding function of the oxytocin receptor. Green: 'allowed' modifications, i.e., modifications that lead to cholesterol derivatives which support the high-affinity binding state of the oxytocin receptor. For example, an aliphatic side chain (-CH_3 or -C_2H_5) at C24 is 'allowed.' Red: 'forbidden' modifications, i.e., modifications that lead to 'inactive' steroids which do not support the high-affinity binding function of the oxytocin receptor. For example, in contrast to a keto function either at C7 or C22, a hydroxyl function at these positions is 'forbidden.' Blue: the hydroxyl function at C3 in β-configuration is absolutely necessary for the high-affinity binding state of the oxytocin receptor. Green circle with a red rim: 6-ketocholestanol is intermediate and leads to 22% [^{3}H]oxytocin binding compared to cholesterol.

cholesterol (47–58%). The high efficiencies of 4-cholesten-3-one (88%) and cholesteryl ethyl ether (67%, low incorporation rate of 45%) demonstrate that the 3-OH group is not necessary for restoring the capacity of cholecystokinin-binding sites.

We also investigated the relationship between receptor ligand binding function and fluidity in plasma membranes containing various steroids as cholesterol substitute. Overall, the cholecystokinin-binding activities were significantly more correlated to the fluidity of the membranes than the oxytocin binding data, i.e., steroids which—like cholesterol—restored the rigidity of the membrane led to the highest binding activity of the CCK_BR. This correlation should be regarded as rather high in view of the fact that the incorporation of the various steroids in cholesterol-depleted membranes not only affects the membrane fluidity but also a variety of other properties of the bilayer such as thickness, curvature, and dipole potential which could influence protein function to a certain degree. In the case of the OTR, we found steroids (e.g., campesterol) which led to high binding activities at low anisotropy values (= high membrane fluidity) and steroids which were completely inactive but yielded intermediate membrane fluidities (e.g., epicholesterol). In addition, we treated HEK-OTR and HEK-CCK_BR plasma membranes with methyl-β-cyclodextrin for various times to remove defined amounts of cholesterol, thus obtaining membranes with different fluidities. In the pretreated membranes, the parameters 'anisotropy' and 'ligand-receptor binding' were measured and correlated (fig. 5). For the CCK_BR, ligand binding increased linearly with the anisotropy values in the pretreated membranes. In contrast, the ligand-binding activity of the OTR declined sharply when the cholesterol amounts were reduced below a critical level (about 57% of the cholesterol content found in untreated membranes). Thus, cholesterol might affect the ligand-binding activity of the OTR via a cooperative mechanism. Hill analysis of cholesterol content versus [^{3}H]oxytocin binding suggests that the

OTR binds several molecules of cholesterol ($n \geq 6$) in a positive cooperative manner. Alternatively, at a critical cholesterol level, the distribution of the cholesterol molecules in the vicinity of the receptor could change significantly, e.g., by alterations in the formation of cholesterol dimers or by rearrangement of cholesterol between both leaflets of the membrane bilayer.

Furthermore, we tested whether the cholesterol-dependent differential behavior of both receptors is also present at the level of receptor signaling. When the cells were depleted of cholesterol, the dose-response curve for oxytocin-activated inositol phosphate production in HEK-OTR cells was shifted to 100- to 1000-fold higher oxytocin concentrations, demonstrating that the low-affinity OTR is physiologically 'active.' In contrast, the dose-response curve for HEK-CCK_BR cells remained unchanged upon cholesterol depletion but the maximal response was reduced by 50% which corresponds to the observed 50% loss of binding sites.

Taken together, these results suggest that cholesterol can modulate the function of neuropeptide receptors by two distinct mechanisms: by changes in membrane fluidity as demonstrated for the human cholecystokinin type B receptor and/or by a highly specific molecular interaction as shown for the human oxytocin receptor. The relationship of high- to low-affinity state(s) of the OTR might reflect its distribution in cholesterol-rich and cholesterol-poor microdomains of the plasma membrane. This assumption was confirmed by recent studies of our group using the human OTR tagged with a mutant green fluorescent protein (OTR-EGFP) which allowed quantification of the total amount of receptor in cholesterol-rich versus cholesterol-poor domains of the plasma membrane of HEK293 cells in relation to its ligand-binding behavior [90]. Although the total number of receptors relative to the total amount of protein was the same in the cholesterol-rich caveolin-containing fraction and in the cholesterol-poor membrane fraction, we found that in the cholesterol-rich fraction the high-affinity OTRs were twofold enriched compared with the quantities of high-affinity receptors in cholesterol-poor membranes. Moreover, OTRs in cholesterol-poor membranes were markedly less stable at 37 °C than receptors in cholesterol-rich membranes. Addition of cholesterol after substantial thermal denaturation led to a partial rescue of binding activity in both membrane domains. In general, addition of cholesterol enhanced the thermal stability of OTR-EGFP in both membrane fractions. Interestingly, solubilized OTRs are also significantly more stable in the presence of cholesterol.

What could be the physiological significance of these observations? In 1987, Murphy and Dwarte [91] reported an increase in the cholesterol concentration of rat myometrial plasma membrane during pregnancy. Since the OTR is expressed in this tissue and might induce labor by contraction of smooth muscle cells, an increase in cholesterol could lead to an enhanced stability of the OTR and to an increase in the amount of OTR in the high-affinity state. This is especially important in view of the fact that the physiological concentration of oxytocin in the blood is very low (in the picomolar range), which means that the responsiveness to oxytocin can only be mediated by receptors at the highest affinity state. Therefore, changes in cholesterol content and distribution in myometrial cells together with an enhanced expression of the OTR at the end of pregnancy [92] may contribute to a strong oxytocin response to induce labor.

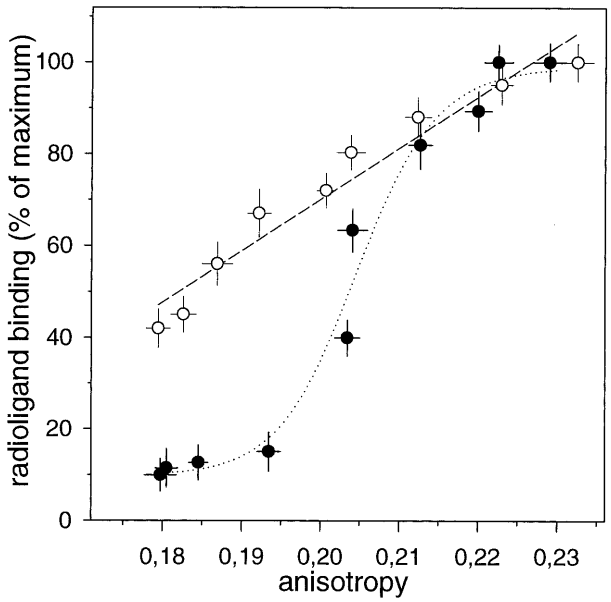

Figure 5. Ligand-binding function of the oxytocin receptor (●) and the cholecystokinin receptor (○) as a function of fluorescence anisotropy in membranes of HEK-OTR and HEK-CCK_BR cells. Plasma membranes of HEK-OTR (●) and HEK-CCK_BR (○) cells were pretreated with methyl-β-cyclodextrin for various times (1–30 min) to remove defined amounts of cholesterol. Hill analysis of cholesterol content versus [^{3}H]oxytocin binding suggests that the OTR binds at least six molecules of cholesterol in a positive cooperative manner. Reprinted with permission from Gimpl et al. [83]. Copyright (1997) American Chemical Society.

The galanin receptor

Galanin is a neuropeptide (29–30 amino acids) in the peripheral and central nervous system. In the periphery, galanin inhibits insulin release induced by glucose and regulates digestive functions such as gastric acid secretion. In the central nervous system, galanin stimulates feeding behavior and release of growth hormone [93].

Very recently, Pang et al. [94] performed experiments with the subtype 2 galanin receptor (GalR2) using the same methods we have employed for the oxytocin and the cholecystokinin receptor. They found that removal of membrane cholesterol by cyclodextrin or culturing of cells in medium supplemented with lipoprotein-deficient serum reversibly reduced galanin affinity for its receptor. Hill analysis suggests that several molecules of cholesterol ($n \geq 3$) could bind in a cooperative manner to the GalR2 compared to at least six molecules of cholesterol which may bind to the OTR. Fluidizing the membranes with 2% ethanol did not affect [^{125}I]galanin binding to membranes of Chinese hamster ovary (CHO) cells expressing the GalR2. In contrast, treatment of the membranes with filipin or cholesterol oxidase, which do not change membrane fluidity, decreased [^{125}I]galanin binding. These results are similar to those we obtained with the OTR [83]. Replenishment of cholesterol-depleted membranes with different steroids [cholesterol, 5-cholestene, 5-pregnen-3β-ol-20-one (= pregnenolone), 4-cholesten-3-one, and 5-cholesten-3-one] suggests specific structural elements in cholesterol that are critical for its interaction with GalR2. It is interesting that 5-cholesten-3-one but not 4-cholesten-3-one or 5-cholestene is able to restore completely the binding activity of cholesterol-depleted GalR2/CHO membranes. This would mean that the 3β-hydroxyl function of cholesterol may be replaced by a 3-keto function without affecting the binding activity of GalR2. This is in contrast to the OTR which needs a 3-hydroxyl function in β-configuration. In addition, the Δ^5 double bond of the ring system is necessary for the GalR2 and may not be replaced by a Δ^4 double bond. Like the OTR, the GalR2 seems to need the presence of the aliphatic tail of cholesterol for proper function, as indicated by the lack of binding activity in the presence of 5-pregnen-3β-ol-20-one. However, for lack of fluidity measurements of membranes treated with ethanol, filipin, and cholesterol oxidase, and of membranes replenished with different steroids, the data of Pang et al. are difficult to interpret.

Taken together, both the GalR2 and OTR may be examples for GPCRs which interact directly with cholesterol. Further steroids have to be tested for their ability to restore galanin-binding activity in order to compare the structure requirements of the GalR2 with those of the OTR.

Other GPCRs

Due to the high cholesterol:protein ratio in plasma membranes, all proteins of the plasma membrane can be assumed to come into contact with cholesterol. Therefore, we compared the cholesterol dependence of different GPCRs by measuring ligand-induced calcium responses in cells depleted of cholesterol. The following cell lines were used in this study: HEK293 cells both untransfected and transfected with either the human OTR (HEK-OTR) or the human V_1 vasopressin receptor (HEK-V_1R) or the human CCK_BR (HEK-CCK_BR), and human astrocytoma cells (1321N1). HEK293 cells endogenously express the M_3 muscarinic acetylcholine receptor (mAChR), the B_2 bradykinin receptor, an adrenergic receptor, and an angiotensin receptor, whereas 1321N1 cells express endogenously the mAChR, the H_1 histamine receptor, and the B_2 bradykinin receptor. Figure 6 shows the ligand-induced calcium responses at different cholesterol contents of the cells. For all receptors investigated, the ligand-induced calcium response of cyclodextrin-treated cells was more or less reduced compared with control cells. This effect was reversible after reloading the cells with cholesterol using cholesterol-methyl-β-cyclodextrin complexes (data not shown).

The effect of cholesterol depletion on ligand-induced calcium responses depends to a certain degree on the cell line investigated. For example, in HEK-OTR cells, reduction of cellular cholesterol to about 40% of control cells had no effect on the acetylcholine-induced calcium response. In contrast, the calcium response of the mAChR in HEK-V_1R cells was reduced to 60% of control. The CCK_BR, the histamine H_1 receptor, and the V_1R showed similar sensitivities to changes in the cellular cholesterol content compared with the acetylcholine receptor. The oxytocin-induced calcium response was slightly more sensitive. The calcium responses of the bradykinin receptor and the adrenergic receptor were clearly more sensitive than the acetylcholine-induced response. The angiotensin receptor was the most sensitive GPCR investigated. Lowering the cholesterol content to 50% almost completely abolished the angiotensin-induced calcium response. Depletion of cellular cholesterol to less than 40% of controls led to marked morphological changes of the cells.

Based on these results, one might speculate that cholesterol enrichment of cells can increase calcium signaling. Experiments with 1321N1 cells support this assumption, at least for the H_1 histamine receptor and the B_2 bradykinin receptor. For cholesterol enrichment, the cells had been incubated with cholesterol-methyl-β-cyclodextrin complexes. Increasing the cholesterol content to 140% of control cells led to a twofold increase in the bradykinin-induced and to a 1.4-fold increase in the histamine-induced calcium response, whereas the acetylcholine-induced calcium response remained unchanged (data not shown).

These data suggest that many GPCRs need cholesterol for optimal signal transduction (at least those which are coupled to intracellular calcium signaling). This is

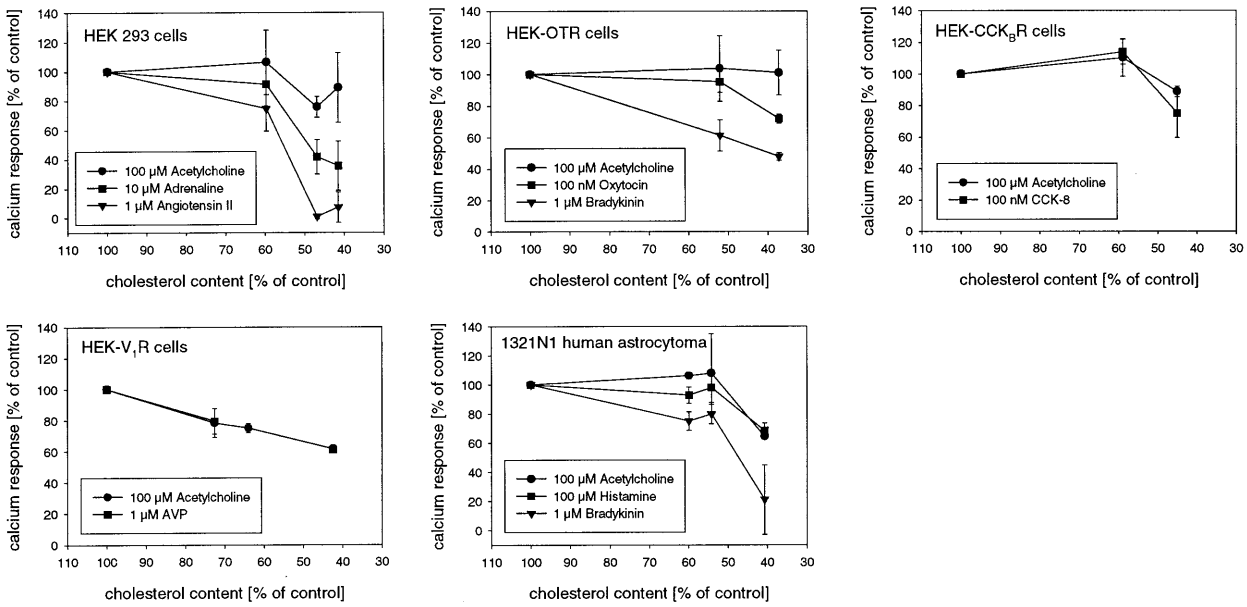

Figure 6. Cholesterol depletion of cells reduces the ligand-induced calcium response of GPCRs. Ligand-induced calcium responses of HEK293 cells both untransfected (HEK293) and transfected with either the human cholecystokinin receptor subtype B (HEK-CCK_BR), the human oxytocin receptor (HEK-OTR), or the human V_1 vasopressin receptor (HEK-V_1R), and human astrocytoma cells (1321N1) were measured at different cellular cholesterol contents. The cellular cholesterol content was reduced by treatment of cells with 10 mM methyl-β-cyclodextrin for various times.

inaccordance with the fact that a fraction of many GPCRs is localized in caveolae, i.e., cholesterin-rich domains of the plasma membrane. In caveolae, calcium signaling could be enhanced by cholesterol and modulated by caveolin. As already mentioned, caveolin is a structural protein of caveolae that binds to cholesterol [15] and possibly to proteins of the signaling cascade [18]. Whether or not caveolin might influence a ligand-induced calcium signal remains to be elucidated.

Measurement of ligand-induced calcium responses of cyclodextrin-treated cells provides no information on whether a receptor interacts directly with cholesterol. Recent ligand-binding studies with V_1 and V_2 vasopressin receptors in isolated membranes indicate that these receptors could also be directly influenced by cholesterol.

Concluding remarks

In the last 20 years, considerable progress has been made in the field of lipid-protein interaction. Lipids are no longer regarded as passive structural elements of biological membranes but as factors that influence the function of many transmembrane proteins. In the case of regulation of receptor function by cholesterol, several different modes of interaction have been found. There are receptors like the human cholecystokinin receptor that are influenced indirectly by cholesterol due to changes in the biophysical properties of the membrane, while other receptors interact directly with cholesterol. Direct interaction can lead to either an increase or decrease in receptor function. Cholesterol is necessary for the high-affinity state of the oxytocin and the galanin receptor and for the ion-gating activity of the nicotinic acetylcholine receptor. In contrast, in the case of rhodopsin, cholesterol impairs receptor function. Some receptors have very stringent requirements for the structure of the cholesterol molecule (e.g., the oxytocin receptor), whereas in the case of the nicotinic acetylcholine receptor, cholesterol can be replaced by steroids which are rather dissimilar to cholesterol (e.g., vitamin D_3). In addition, cholesterol can enhance receptor stability, as seen for example with the oxytocin receptor and rhodopsin. Further studies should provide more detailed information on cellular cholesterol distribution and on the molecular mechanisms of cholesterol-receptor interaction. Diseases connected with disorders in cholesterol metabolism or intracellular cholesterol transport, like Smith-Lemli-Opitz syndrome, Niemann-Pick type C, or Tangier disease, emphasize the need for better knowledge about the impact of cholesterol on cellular processes and membrane proteins.

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